

CCL9 INDUCED BY TGF- β SIGNALING IN MYELOID CELLS
ENHANCES TUMOR CELL SURVIVAL IN THE
PREMETASTATIC LUNG

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ABSTRACT

The majority of cancer patients die from metastasis. To achieve metastasis, tumor cells must first survive and then proliferate to form colonies. Compelling data have shown the indispensable participation of host microenvironment for metastasis. Bone marrow derived myeloid cells sculpt a tumor-promoting microenvironment in the premetastatic organs prior to tumor cell arrival. However, the molecular mechanisms for this “seed and soil” hypothesis are unclear.

Here we report that CCL9 was significantly produced and secreted by Gr-1+CD11b+ cells when co-cultured with tumor cells, and in the premetastatic lung. CCL9 knockdown (KD) in myeloid cells decreased metastasis, and this process signaled through its sole receptor CCR1. Overexpression of CCR1 lost the metastasis-promoting function in the context of CCL9 KD. CCL9 enhanced tumor cell survival in the premetastatic organs. The underlying molecular mechanisms included activation of cell survival factors phosphorylated AKT and BCL-2, as well as inhibition of Poly (ADP-ribose) polymerase (PARP)-dependent apoptosis pathway. Additionally, CCL9/CCR1 had autocrine effects, which enhanced CCL9 secretion and the survival of Gr-1+CD11b+ cells.

We found that CCL9 was a key effector of myeloid transforming growth factor β (TGF- β) pathway that promotes metastasis. Decreased metastasis in mice with myeloid specific TGF- β receptor II deletion (Tgfr2^{MyeKO}) correlated with lower CCL9 expression in TGF- β deficient myeloid cells. Importantly, CCL9 over-expression in these cells

rescued metastasis deficiency. Furthermore, inhibition or KD of p38, a downstream molecule in TGF- β signaling pathway, decreased CCL9 expression in wild type but not T β RII-deficient Gr-1⁺CD11b⁺ cells.

Our findings likely had clinic significance as human data paralleled those from mouse studies. CCL23, the human orthologue for mouse CCL9, was elevated in the supernatant by peripheral blood mononuclear cells (PBMCs) when co-cultured with human tumor cells. Publicly available databases showed a negative correlation between CCL23/CCR1 expressions and metastasis free survival of cancer patients. Our work indicates that CCL9 may serve as a target for treating cancer patients with metastasis. This approach may bypass the complexity of TGF- β treatment that results from its dual pro- and anti-cancer function.

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LIST OF ABBREVIATIONS

4T1-luc	Luciferase labeled 4T1 cells
ADAM	A disintegrin and metalloproteinase domain-containing protein
AKT	Protein kinase B
ANOVA	Statistical analysis of variance
ATCC	American type culture collection
APC	Antigen presenting cells
BALB/c	One of the inbred mouse strain
BAX	BCL2-associated X protein
BCL-2	B-cell lymphoma 2
BLAST	Basic Local Alignment Search Tool
BM	Bone marrow
BMPRII	Bone morphology protein receptor II
C56BL/6	One of the inbred mouse strain
CCL	Ligand for CC chemokine receptor
CCR1	CC chemokine receptor type 1
CTC	Circulating tumor cell
CXC	Ligand for CXC chemokine receptor
CXCR	CXC chemokine receptor
DAPI	A fluorescence dye for nuclear staining

DC	Dendritic cell
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DN	Dominant negative
EMT	Epithelial to mesenchymal transition
FACS	Fluorescence-activated cell sorting
HER2	Human epidermal growth factor receptor 2
HGF	hepatocyte growth factor
IF	Immunofluorescence Staining
IFN- γ	Interferon-gamma
IHC	Immunohistochemistry staining
iMCs	Immature myeloid cells
iNOS	Inducible nitric oxide synthase
JHU	Johns Hopkins University
KO	Knockout
KOMP	Knockout Mouse Project
LOX	Lysyl oxidase
MET	Mesenchymal to epithelial transition
MC26	Colorectal cancer cell line
MDSCs	Myeloid derived suppressor cells

MIP	Macrophage inflammatory protein
MISC	Myeloid immune suppressor cells
MMP	Matrix metalloproteinase
MRP-2	Macrophage inflammatory protein-related protein-2
MSCs	Mesenchymal stem cells
NCI	National Cancer Institute
NeuAB	Neutralizing antibody
NIH	National Institutes of Health
NK	Natural killer cells
OPG	Osteoprotegerin
PAM50	Prediction analysis of microarray using 50-gene classifier
PARP	Poly (ADP-ribose) polymerase
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphoinositol-3 kinase
PuMA	Ex vivo pulmonary metastasis assay
PyMT	Polyomavirus middle T antigen
RANKL	Receptor activator of NF- κ B ligand
RANTES	Regulated on activation, normal T cell expressed and secreted

Rb	Retinoblastoma protein
rmCCL9	Recombinant mouse CCL9
ROS	Reactive oxygen species
S100A8/9	S100 calcium binding protein A8/9
SCVM	Single cell videomicroscopy
SDF-1	Stromal derived factor 1
SE	Standard error
SMAD	Downstream molecules of the canonical TGF- β pathway
TCR	T cell receptor
TGF- β	Transforming growth factor beta
Treg	Regulatory T cells
TUNEL	Terminal deoxynucleotidyl transferase dUTP
T β RI	TGF- β receptor I
T β RII	TGF- β receptor II
T β RIII	TGF- β receptor III
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VEGFR1	VEGF receptor 1
WHO	World Health Organization

CHAPTER I

INTRODUCTION

Metastasis cascade

Metastasis is a question of life or death. Cancer is now the leading cause of death. According to the newest report from the World Health Organization (WHO), there were 8.2 million cancer-related deaths in 2012 worldwide. It is estimated that there were 1,665,540 new cases of cancer and 585,720 deaths caused by cancer in the United States in 2014 (Siegel et al., 2014). Metastasis is an ominous feature of tumor progression and accounts for over 90% of cancer-associated deaths (Mehlen and Puisieux, 2006). Thus, understanding and developing methods to target metastasis is important for effective therapies of cancer patients.

Metastasis is a process in which tumor cells spread from a primary tumor of origin and colonize to the distant organs. The metastasis cascade often occurs in the following order (Fig. 1). (1) Local invasion. Tumor cells must acquire the ability to migrate and invade in order to break away from the primary tumor site. These abilities are affected by genetic and epigenetic changes in tumor cells, as well as the influence of the tumor microenvironment. (2) Intravasation. Tumor cells enter blood vessels, which are established through tumor vascularization. This step normally involves epithelial to mesenchymal transition (EMT). (3) Circulation. Cancer cells traveling through the circulatory system are dubbed circulating tumor cells (CTCs). CTCs circulate through blood vessels until they reach the secondary organ. They display properties of

anchorage-independent survival. (4) Extravasation. CTCs exit the blood vessels once successfully lodge in the distant tissue and subsequently invade the foreign tissue. Tumor cell mesenchymal to epithelial transition (MET) is critical for extravasation. It has been shown that CTCs can also proliferate inside of the microvessels (Wong et al., 2002). (5) Colonization. Tumor cells proliferate in the distant organ and form tumor nodules.

Tumor-stroma interactions are indispensable participants in the metastatic process. Of interest, cancer cells often do not need additional genomic alterations in order to establish metastatic colonies when compared to the cancer cells at the primary tumor site (Vanharanta and Massague, 2013). In fact, gene expression orchestrated by microenvironmental cues play a fundamental role in the metastatic processes. Myeloid cells have been implicated in supporting cancer metastasis (Grivennikov et al., 2010; McAllister and Weinberg, 2010). The functionality of these cells involves suppression of host anti-tumor immunity (Gabrilovich et al., 2012; Pang et al., 2013), promotion of tumor angiogenesis (Yang et al., 2004), enhanced tumor cell extravasation (Qian et al., 2011), migration and invasion (Kitamura et al., 2007), and premetastatic niche formation (this will be further discussed.) (Gil-Bernabe et al., 2012; Kaplan et al., 2005).

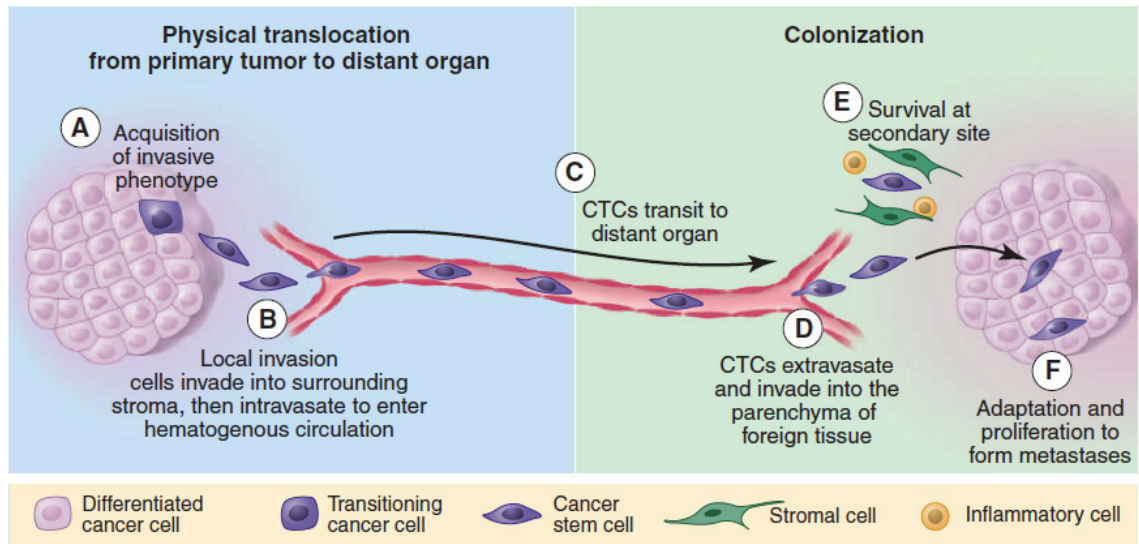


Figure 1. The metastasis cascade.

Metastasis can be envisioned as a process that occurs in two major phases: (i) physical translocation of cancer cells from the primary tumor to a distant organ and (ii) colonization of the translocated cells within that organ. **(A)** To begin the metastatic cascade, cancer cells within the primary tumor acquire an invasive phenotype. **(B)** Cancer cells can then invade the surrounding matrix and move toward blood vessels, where they intravasate to enter the circulatory system. **(C)** CTCs circulate through blood vessels and arrive at the distant organ, which serves as their primary means of passage to distant organs. **(D)** At the secondary organ, CTCs exit the blood vessels and invade the local tissues of a hostile microenvironment. **(E)** At the distant organ, cancer cells must be able to evade the innate immune response and to survive as a single cell or as a small cluster of cells. **(F)** Cancer cells adapt to the foreign microenvironment and initiate proliferation to develop into an active macrometastatic deposit. Figure adapted from Chaffer & Weiberg, Science. 2011 Mar 25;331(6024):1559-64. doi: 10.1126/science.1203543.

Tumor cell survival is a rate-limiting step in metastasis

Tumor cell survival is a rate-limiting step in cancer metastasis. Tumor cells must firstly evade apoptosis, in order to survive and proliferate in the distant organ. This is supported by the fact that in human patients, the expression of pro-apoptotic Bcl-2-associated X protein (BAX) negatively correlates with metastasis, whereas anti-apoptotic protein B-cell lymphoma-2 (BCL-2) positively associates with metastasis (Krajewski et al., 1995; Le et al., 1999). Highly metastatic tumor cells also exhibit a significantly lower level of apoptosis in the distant organ compared to none or low metastatic tumor cells (Fidler, 1975; Hong et al., 2012), and loss or down regulation of apoptosis directly impacts metastasis in different cancers (Cotter, 2009; Lowe and Lin, 2000). Blocking tumor cell apoptosis increases metastasis (Del Bufalo et al., 1997; Owen-Schaub et al., 1998), whereas promoting tumor cell apoptosis decreases metastasis (Medina-Ramirez et al., 2011; Owen-Schaub et al., 1998). Additionally, activation of the cell survival molecules, such as Src or AKT, directly inhibits tumor cell apoptosis and promotes tumor cell survival (Zhang et al., 2009). Targeting tumor cell apoptosis is a promising strategy for cancer treatment (Fesik, 2005).

Despite the fact that apoptotic molecules within tumor cells play fundamental roles in tumor cell survival, recent studies have found that host cells also contribute to increased tumor cell survival (Lazennec and Richmond, 2010; Steeg, 2006). For example, bone marrow stromal cells isolated from cancer patients secrete osteoprotegerin (OPG),

which protects breast cancer cells from TRAIL-induced apoptosis (Neville-Webbe et al., 2004). In mouse models, macrophages express $\alpha 4$ integrin (Chen et al., 2011) and Gr-1+CD11b+ cells secrete S100A8/9 (Acharyya et al., 2012) to inhibit tumor cell death at the metastatic site.

Thus, identifying factors responsible for tumor cell survival, especially those from host cells, may provide an important insight for understanding cancer biology and facilitating the design of effective cancer treatments.

Microenvironment in the premetastatic organ

The microenvironmental cue in a distant organ (premetastatic organ) is critical for metastatic colony establishment. Premetastatic lung is the lung microenvironment prior to the arrival of tumor cells. Tumors modulate the premetastatic lung environment and build up a “soil” that is favorable to the seeding of tumor cells. Developing a premetastatic tissue includes changes in inflammatory responses, matrix remodeling, and other bioactive oncogenic molecules, such as vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) (Kaplan et al., 2005). The “seed and soil” hypothesis is firstly proposed by Steven Paget, who theorizes that the congenial microenvironment is required for malignant tumor cells to colonize in the secondary organ (Paget, 1989). In the battle with cancer, significant efforts have been devoted to understanding the behavior of tumor cells. Cancer biologists have successfully identified the important roles of tumor

suppressor genes such as p53 and retinoblastoma protein (Rb), as well as oncogenes such as myc and Src. However, targeting these molecules in tumor cells has only succeeded in a portion of cancer patients, and this may be due to individualized host immunity. As a result, understanding the “soil” of the tumor microenvironment is greatly needed, as it may shed insight into new methods of cancer treatment as an addition to the current ones.

Hematopoietic cells, especially myeloid cells, play a key role in the premetastatic organs. For example, bone marrow-derived hematopoietic progenitors that reside in the premetastatic lung secrete high levels of VEGF, which attracts VEGF receptor 1 expressing (VEGFR1+) myeloid cells to form a premetastatic niche for the incoming tumor cells (Kaplan et al., 2005). Mac-1+ myeloid cells are recruited by S100A8/9 (S100 calcium binding protein A8/9) in the premetastatic lung to promote tumor migration and invasion (Hiratsuka et al., 2006). Also, CD11b+ cells are recruited by Lysyl oxidase (LOX) that is secreted by hypoxic tumor cells, and in response to produce matrix metalloproteinase-2 (MMP2) to enhance tumor cell invasion by cleaving collagen (Erler et al., 2009). In the premetastatic brain, Gr-1 neutralization decreases the myeloid cell population and the subsequent brain metastasis (Liu et al., 2013).

Our research confirms the supportive function of hematopoietic cells in metastasis and demonstrates that a specific myeloid cell subset, Gr-1+CD11b+ cells of tumor-bearing mice, build up an inflammatory, proliferative, and immune suppressive premetastatic lung microenvironment (Yan et al., 2010). Gr-1+CD11b+ cells sculpt the

lung by altering the expression profile of cytokines. We have found reduced type I cytokine interferon-gamma (IFN- γ) and increased expression of type II cytokines (Yan et al., 2010). Additionally, we have observed an increased MMP9 expression in the premetastatic lung, which leads to blood vessel leakage, and facilitates tumor cell extravasation (Yan et al., 2010).

However, whether myeloid cells secrete cytokines into the premetastatic lung microenvironment to promote tumor cell survival and the consequential lung metastasis need to be carefully investigated.

Gr-1+CD11b+ cells promote metastasis

Gr-1+CD11b+ cells are also known as Myeloid Immune Suppressor cells (MISCs) or Myeloid Derived Suppressor cells (MDSCs). In mice, it is a heterogeneous cell population expressing granulocyte marker Ly6G, monocyte marker Ly6C, and macrophage marker F4/80, and all of these cells express the macrophage lineage marker CD11b. In cancer patients, immature myeloid cells typically express myeloid markers CD11b/CD33/CD34. These cells do not express the lymphocyte marker CD14 or leucocyte antigen HLA-DR, and can vary in their expression of CD15 and other markers (Ostrand-Rosenberg and Sinha, 2009).

The Gr-1+CD11b+ cell type is a good model to study the function of immature myeloid cells under cancer conditions. In humans, immature myeloid cells are

overproduced in the peripheral blood of patients with neck/head, breast, and lung cancer (Almand et al., 2001). In mice, Gr-1+CD11b+ cells are significantly increased in the spleen and bone marrow of tumor-bearing mice and become the main population of immature myeloid cells (Youn et al., 2008). Under tumor conditions, these myeloid cells are prevented from further differentiating into mature macrophages or dendritic cells (DCs) to exert immune responses. Instead, they remain in their immature status and become immune suppressive (Gabrilovich and Nagaraj, 2009).

Gr-1+CD11b+ cells are well characterized for their function in modulating the immune response (Fig. 2). They are known to regulate T cell function through following four mechanisms. (1) Down regulating TCR (T cell receptor) complex and T cell proliferation by secreting arginase and iNOS (inducible nitric oxide synthase) to deplete L-arginine as an important nutrient for T cells (Bronte and Zanovello, 2005). (2) Inhibition of normal cytokine production from T cells by producing ROS (reactive oxygen species) like H₂O₂ (Schmielau and Finn, 2001). (3) Depletion of cysteine that is essential for T cell activation (Srivastava et al., 2010). (4) Activation/expansion of Treg (regulatory T cell) populations (Pan et al., 2010). Gr-1+CD11b+ cells also interfere with lymphocyte trafficking and viability by expressing ADAM17 (disintegrin and metalloproteinase domain-containing protein 17), which cleaves L-selectin on the naive CD4+ and CD8+ T cells (Schmielau and Finn, 2001).

Other than T cells, Gr-1+CD11b+ cells diminish the cytotoxicity of natural killer (NK) cells by inhibiting perforin production (Liu et al., 2007). They also inhibit Th1

cytokine IL-12 production from macrophages by secreting high level of Th2 cytokine IL-10 (Sinha et al., 2007). Additionally, Gr-1+CD11b+ cells inhibit dendritic cell differentiation by S100A9 production, thus diminish the presence of APC (antigen presenting cells) (Cheng et al., 2008).

In addition to their immune modulating function, Gr-1+CD11b+ cells directly promote tumor metastasis. Those cells are found at the tumor invasive front and secrete high levels of VEGF to enhance metastasis by promoting tumor angiogenesis (Yang et al., 2004). Gr-1+CD11b+ cells are also recruited in the premetastatic lung to build up a tumor-favorable lung microenvironment (Yan et al., 2010). However, the mechanisms by which Gr-1+CD11b+ and tumor cells cooperatively interact for metastasis need to be addressed.

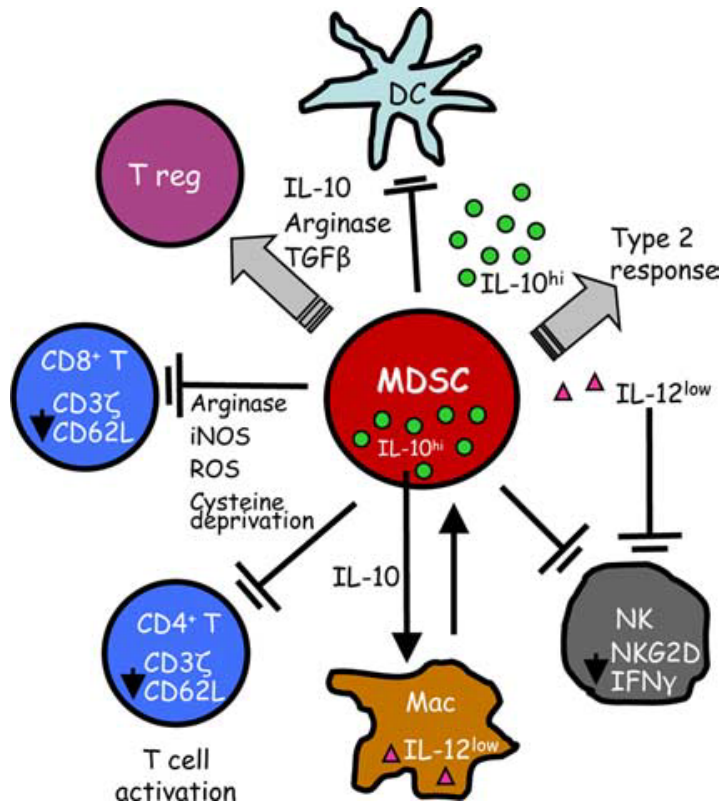


Figure 2. Gr-1+CD11b+ cells suppress anti-tumor immunity through a variety of mechanisms.

T cell activation is suppressed by cysteine deprivation, Treg induction, as well as arginase, iNOS, and ROS produced by Gr-1+CD11b+ cells. Innate immunity is impaired by the suppression of NK cell cytotoxicity, and by the up-regulation of IL-10, which in turns decreases macrophage produced IL-12. Antigen presentation is limited by the expansion of Gr-1+CD11b+ cells at the expense of DCs. MDSCs: Gr-1+CD11b+ cells; NK: natural killer cells; DC: dendritic cells, Treg: T regulatory cells. Figure adapted from Ostrand-Rosenberg and Sinha, J Immunol. 2009 Apr 15;182(8):4499-506. doi: 10.4049/jimmunol.0802740.

4T1 mammary tumor and B16 melanoma study models

In this study, we focus on two tumor models: breast cancer and melanoma. Breast cancer is the fifth most common cancer causes deaths worldwide. In the United States, according to 2014 estimate, breast cancer has the highest incidence (29%) and it is the second most common cause of death (15%) in females. Melanoma has the 5th highest incidence (14%) and it is the 8th most common cause of death (2%).

The 4T1 mammary tumor and B16 melanoma models share many characteristics with human breast cancer and melanoma, respectively, particularly in their ability to metastasize to the lungs. The 4T1 model includes four isogenic tumorigenic lines, all of which originates from one spontaneous murine mammary gland tumor in a BALB/cfC3H mouse (Dexter et al., 1978). Ordered from lowest to highest metastatic capability, they are the 67NR, 168FARN, 4TO7 and 4T1 cell lines. 67NR forms primary tumors; 168FARN disseminates from the primary tumors and can form metastasis in lymph nodes; 4TO7 metastasizes to the lungs but does not form colonies; and 4T1 can finish all steps and form metastatic nodules mainly in the lung but also in other organs including the bone and brain (Aslakson and Miller, 1992).

The B16 model includes 11 isogenic tumorigenic cell lines number sequentially from B16F1, B16F2... to B16F11, all of which originate from one spontaneous murine melanoma in a C57BL/6 mouse. It has been determined that cell lines of later passages have higher metastatic capabilities compared to earlier passages (Fidler, 1975). To produce these cell lines, cells were injected into animals through the tail vein and

pulmonary nodules were collected 3 weeks later. Harvested cells were then cultured until confluent and injected back into mice. This procedure was repeated 10 times, with B16F1 representing the line originating from the first passage and each consecutive passage labeled with the succeeding number (Fidler, 1975).

The B16BL6 cell line was generated by injecting B16F10 cells into the urinary bladders of C57BL/6 mice. The bladders were collected and cultured on a semi-solid agar *in vitro*. Tumor cells that invade into the agar through the bladder wall were then recovered and established as a cell line. This was repeated 8 times, with B16BL1 representing the first passage and each additional passage numbered consecutively. Compared with B16F10, B16BL6 exhibited a higher invasive capability and an increased frequency of spontaneous metastasis (Poste et al., 1980). In this study, we will use melanoma cell lines B16F1, B16F10, and B16BL6.

Transforming growth factor beta (TGF- β): pro-/anti-tumor functions and pathways

TGF- β acts both as a tumor suppressor and a tumor promoter. TGF- β signaling is a master regulator for a number of biological processes. In normal cells, TGF- β is important for cell growth, differentiation, proliferation, and migration. Under tumor conditions, TGF- β regulates tumor initiation, progression, and metastasis (Bierie and Moses, 2006). Alterations of TGF- β receptor I (T β RI), T β RII, and the SMADs are found in multiple human cancers. These alterations include deletion, mutation, down regulation of molecules that activate this pathway, or up regulation of molecules that inhibit this pathway (Levy and Hill, 2006). In mouse models, genetic deletion or knockdown of the TGF- β receptors and SMADs often results in a more malignant tumor phenotype (Yang et al., 2010). However, TGF- β also exhibits a tumor-promoting function. High TGF- β activity of glioma cells associates with the poor prognosis of patients (Bruna et al., 2007). Overexpression of T β RI or TGF- β 1 accelerates metastasis in the PyMT (polyomavirus middle T antigen)-induced mammary tumor-bearing mice (Muraoka-Cook et al., 2004; Siegel et al., 2014).

The TGF- β pathway is activated upon the binding of TGF- β ligand to receptor. There are three TGF- β isoforms: TGF β 1, TGF β 2, and TGF β 3. TGF β 1 is expressed in epithelial, endothelial, hematopoietic, and connective tissue cells; TGF β 2 is expressed in epithelial and neuronal cells; and TGF β 3 is expressed primarily in mesenchymal cells (Achyut and Yang, 2011). TGF- β ligands signal through T β RI, T β RII, and T β RIII. Seven T β RI and five T β RII members have been previously described and they all contain a

serine/threonine kinase domain within the cytoplasmic domain. Both TGF β 1 and TGF β 3 bind T β RII with high affinity whereas TGF β 2 can only bind T β RII (Bierie and Moses, 2006).

The variety of downstream pathways may explain the dual function of TGF- β . TGF- β signaling pathways include both the canonical SMAD-dependent pathway and the non-canonical SMAD-independent pathway. Upon the binding of TGF- β ligand to the T β RII homodimer, T β RI homodimer is recruited and phosphorylated by T β RII. The T β RI and T β RII heteromeric signaling complex recruits and phosphorylates the SMAD2/3 complex, which then binds SMAD4 and translocates into nucleus to regulate gene transcription. Smad7 is a negative regulator that inhibits the phosphorylation of the SMAD2/3 complex (Nakao et al., 1997). In addition, non-canonical TGF- β signaling pathways include phosphoinositol-3 kinase (PI3K), mitogen-activated protein kinase, and small guanosine triphosphatase pathways. These pathways are most often implicated in tumor cell motility and migration (Achyut and Yang, 2011) (Fig. 3).

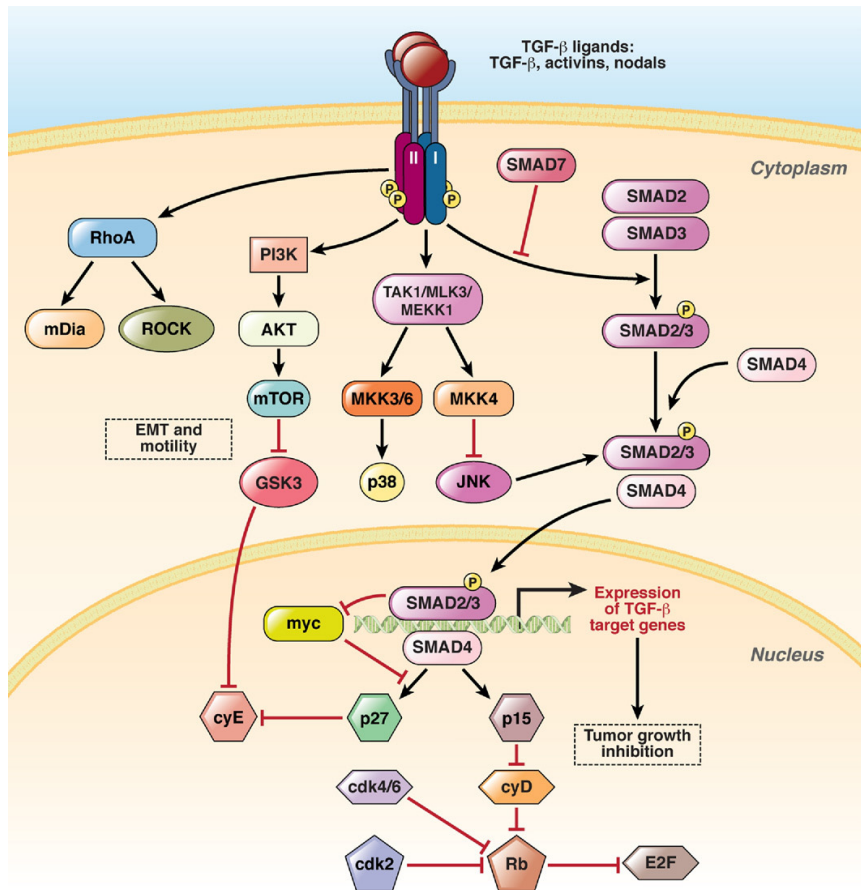


Figure 3. TGF-β pathways.

TGF-β ligands signaling through TGF-β receptors activates the canonical SMAD-dependent pathway. It also activates non-canonical SMAD-independent signaling pathways, such as mitogen-activated protein kinase, PI3 kinase, and small guanosine triphosphatase. These pathways are implicated in EMT, tumor cell motility, and migration. cyD, cyclin D; cyE, cyclin E; GSK3, glycogen synthase kinase 3; MEKK1, mitogen-activated protein kinase kinase kinase 1; MKK4, mitogen-activated protein kinase kinase 4; MLK3, mixed lineage kinase 3; mTOR, mammalian target of rapamycin; myc, myelocytomatosis oncogene; PI3K, phosphatidylinositol 3-kinases; Rb, retinoblastoma; RhoA, ras homolog gene family, member A; ROCK, rho-associated protein kinase; TAK1, TGF-β activated kinase 1. Figure adapted from Achyut and Yang, *Gastroenterology*. 2011 Oct;141(4):1167-78. doi: 10.1053/j.gastro.2011.07.048. Epub 2011 Aug 11.)

TGF- β and metastasis

TGF- β regulates metastasis through different mechanisms. For instance, TGF- β has an immunosuppressive function, particularly, TGF- β activates Treg and inhibits cytotoxic T cells (Blobe et al., 2000; Thomas and Massague, 2005). TGF- β also promotes EMT and inhibits MET (Xu et al., 2009). In addition, TGF- β enhances angiogenesis as neutralization down regulates whereas TGF- β 1 increases blood vessel formation (Tuxhorn et al., 2002).

It has been reported that TGF- β is a cell type-dependent metastasis promoter but can also function as a metastasis suppressor. Large amount of evidence suggest that TGF- β signaling in epithelial cells (Forrester et al., 2005; Kitamura et al., 2007), fibroblasts (Hembruff et al., 2010), and T cells (Kim et al., 2006) serves as a metastasis suppressor, as its deletion in these cell types promotes tumor development and progression. Interestingly, unlike in the cell types discussed above, myeloid specific TGF- β promotes metastasis, as deletion of T β RII in these cells significantly decreases lung colonization (Pang et al., 2013).

Chemokines and cytokines are key effectors in the cell-type dependent TGF- β regulation of tumor metastasis. The chemokine profile of human breast tumors due to a loss of epithelial TGF- β signaling correlates with poor prognosis (Bierie et al., 2009). In mouse models, epithelial TGF- β blocks the production of chemokines that are critical for the recruitment of inflammatory cells to the tumor microenvironment and the resulting carcinoma-immune cell interactions (Bierie et al., 2009; Ijichi et al., 2011; Yang et al.,

2008a). Fibroblast TGF- β suppresses hepatocyte growth factor (HGF) and CCL2 expression, which are important for epithelial cell proliferation and survival (Bhowmick et al., 2004; Hembruff et al., 2010). Loss of TGF- β signaling in T cells promotes the expression of Th2 cytokines, which further promote stromal cell expansion (Kim et al., 2006). On the other hand, myeloid TGF- β inhibits type I cytokine IFN- γ and promotes type II cytokines TGF β 1, arginase1, and iNOS to enhance metastasis (Pang et al., 2013). In addition, TGF- β has global effects in regulating cytokine expression. It enhances the production of chemoattractants S100A8/9 in the premetastatic lung to promote tumor cell migration, as TGF- β neutralization *in vivo* decreases both S100A8/9 and metastasis (Hiratsuka et al., 2006).

CHAPTER II

MATERIALS AND METHODS

Cell lines and cell culture

Murine mammary tumor cell lines 67NR, 168FARN, 4T07, 4T1, 67NR-GFP, 4T1-GFP, 4T1-luciferase, melanoma cell lines B16F1, B16F1-GFP, B16BL6, B16F10, mouse macrophage cell line RAW264.7, and neutrophil cell line 32DCI3 (ATCC CRL-11346), as well as human breast cancer cell lines MDA-MB-231, MDA-MB-435, human melanoma cell lines A375S2, SK-MEL-28, and human lung cancer cell line H460, were maintained per standard cell culture techniques. 67NR-GFP, 4T1-GFP, B16F1, B16F10, B16F1-GFP, A375S2, and SK-MEL-28 were gifts from Drs. Chand Khanna, Glenn Merlino, and Shioko Kimura (NCI). 67NR, 168FARN, 4T07, 4T1, 67NR-GFP, 4T1-GFP, 4T1-luciferase, B16F1, B16F1-GFP, B16BL6, B16F10, RAW264.7 were cultured in DMEM (Gibco, 11995-065). H460, MDA-MB-231, MDA-MB-435, A375S2, and SK-MEL-28 were cultured in RPMI (A10491-01). 32DCI3 was cultured in glucose enriched RPMI (Gibco, 22400) with 0.4 ng/ml IL-3 (Gibco, PMC0034). All culture media contained 10% FBS (Gibco, 10091-148) and 1x Antibiotic-Antimycotic (Gibco, 15240-062). Cells were passaged using trypsin-EDTA (Gibco, 25200-056). All cell lines were incubated at 37°C with 5% CO₂.

Animals

Six to eight week old female BALB/c and C57BL/6 were purchased from NCI Fredrick. $Tgfb\beta 2^{MyeKO}$ and $Tgfb\beta 2^{Floxed}$ control mice were produced as previously described (Pang et al., 2013). In brief, myeloid specific promoter LysM driven Cre was specifically expressed in myeloid cells, which recognized and removed sequence between two LoxP sites in the exon 2 of $Tgfb\beta 2$. p38 dominant negative mice were gifts from Dr. Albert J. Fornace Jr (Georgetown University). All animal studies were approved by the National Cancer Institute Animal Care and Use Committee.

Spontaneous and experimental metastasis models

For spontaneous orthotopic metastasis assays, mammary tumor 4T1 cells (5×10^5) were injected into the #2 mammary fat pad (MFP); B16F10 melanoma cells (1×10^6) were injected subcutaneously. For experimental metastasis assays, 67NR-control/67NR-CCR1 cells (5×10^5 or 1×10^6), 67NR, 4T1 cells (5×10^5), and B16F1-control/B16F1-CCR1 cells (1×10^6) were injected into the tail vein (TVI) of recipient mice. For the co-injection experiment, tumor cells were injected with Gr-1⁺CD11b⁺ (1×10^6 or 1.5×10^6) or RAW264.7 cells (2×10^5).

Primary tumor size and lung metastasis evaluation

Tumor size was measured twice a week using calipers beginning on day 8 after tumor inoculation, and the tumor size was calculated as $\text{Volume} = (\text{Width})^2 \times \text{Length} / 2$.

The amount of lung metastasis was evaluated using a whole lung mounting procedure as previously described (Jessen et al., 2004), by lung weight, or H&E staining of butterfly sections. In brief, lungs were firstly fixed in Formalin (VWR, BDH0502) overnight, dehydrated and rehydrated, and last stained with hematoxylin (Sigma, MHS80). One butterfly section was obtained from each mouse, and at least 5 mice were evaluated for each experimental group.

***In vivo* bioluminescence imaging**

Mice were injected with D-luciferin intraperitoneally (Caliper Life Sciences, 100 μ l of 10 mg/ml) 15 minutes before imaging. Luminescent signals were detected using an IVIS-SPECTRUM *in vivo* photon-counting device (Caliper Life Sciences). Images were quantified as photon counts/second using the Living Image software (Caliper Life Sciences).

Single cell videomicroscopy (SCVM) for evaluating tumor cell survival

Mice were euthanized and lungs were removed 6 hours after tail vein injection of GFP labeled tumor cells (5×10^5) together with Gr-1+CD11b+ (1.5×10^6) or RAW264.7 cells (2×10^5). Images were normalized to the basal signal obtained from lungs 1 hour after tail vein injection. Lungs were observed under the fluorescent microscope. 10 random pictures at 20 \times magnification were taken and analyzed as fluorescence signal per field by OpenLab software (Improvision) or ImageJ. For extravasation experiment, mice

were injected with 100 μ l of 12.5 μ g/ μ l 70,000 MW tetramethylrhodamine (Invitrogen, D1818). The lungs were imaged under a Zeiss 510 NLO confocal microscope. The images were analyzed with Zeiss ZEN software.

***Ex vivo* pulmonary metastasis assay (PuMA)**

GFP labeled tumor cells (5×10^5) were co-injected with sorted Gr-1+CD11b+ cells (1.5×10^6) or RAW264.7 cells (2×10^5) through the tail vein. The mice were euthanized 5 minutes after injection, and their lungs were infused with an agarose medium mixture as previously described (Mendoza et al., 2010). The lung sections (1-2mm thick) were placed on Gelfoam (Pfizer-Pharmacia & Upjohn Co., 09-0315-08) and cultured for 1-2 weeks. LEICA-DM IRB fluorescent inverted microscope (Leica) and Retiga-EXi Fast 1394 Mono Cooled CCD camera (QImaging) were used to capture GFP positive cells at 10 \times or 2.5 \times magnification. The GFP fluorescence was obtained and analyzed using OpenLab software (Improvision) or ImageJ (Mendoza et al., 2010). The fluorescence intensity per field was quantified, normalized to day 0 signal, and presented as metastasis survival index. Three to six lung sections for each mouse and a total of 3-4 mice were evaluated for each experimental group.

Immunofluorescence (IF) staining and TUNEL assay

Paraffin-embedded lung sections or chamber slides with tumor cell culture were incubated with primary antibodies for GFP (Santa Cruz, SC-8334) or PAR (BD

Pharmingen, 550781). Alexa flour 488 or 594 secondary antibodies were used for detection (Invitrogen). For Terminal deoxynucleotidyl transferase dUTP (TUNEL) assay (Roche Applied Science, 11684795910), lungs were removed 6 hours after tail vein co-injection of GFP labeled tumor cells (5×10^5) with Gr-1+CD11b+ (1.5×10^6) or RAW264.7 cells (2×10^5). The lungs were fixed and Paraffin-embedded for sectioning. TUNEL was performed according to the manufacturer's protocol. The slides were then mounted with Prolong Gold antifade reagent with DAPI (Invitrogen, P36931) and examined using fluorescence microscopy.

Single cell suspension

Single cell suspensions were made from spleens, peripheral blood (Yang et al., 2004), and lung tissues (Ljung et al., 1989) of normal and 4T1 tumor-bearing mice. Briefly, spleenocytes were collected by direct smashing with a 3 ml syringe plunger into a 40 μ m cell strainer (BD Pharmingen). The lungs were firstly minced into small pieces, then incubated for 1 hour at 37°C in a digesting cocktail. The digesting cocktail included 1.5 mg/ml collagenase (CD130), 1 mg/ml trypsin (T9201), 0.2 mg/ml DNase (D5025), and 0.0875 mg/ml hyaluronidase (H3506). All reagents were purchased from Sigma and dissolved in serum-free DMEM (Gibco, 11995-065).

Flow cytometry and cell sorting (FACS and MACS)

Cells were labeled with fluorescence-conjugated antibodies: Gr-1, CD11b, Ly6G, Ly6C, F4/80, AnnexinV, 7AAD. All antibodies listed above were purchased from BD Biosciences. CCR1 (FAB5986A) and isotype control (IC013A) were purchased from R&D systems. For flow cytometry analysis, cells were run on a FACS Calibur or Fortessa flow cytometer (BD, San Jose, CA) and analyzed using FlowJo. For sorting, Gr-1+CD11b+ cells, Ly6G+CD11b+ cells, Ly6C+CD11b+ cells, and F4/80+CD11b+ cells were sorted from spleens of 4T1 tumor-bearing mice by FACS Aria flow cytometer (BD) or MACS (Magnetic-activated cell sorting) according to the manufacturer's protocol (Miltenyi Biotec). CD11b (BD Biosciences, 558013) and anti-PE (Miltenyi Biotec, 130-048-801) microbeads were used for MACS. For sorting human CD33+ myeloid cells, normal human whole blood was obtained from the NIH blood bank in the clinical center. Myeloid cells were enriched by Ficoll-PaqueTM (17-1440-02, GE Healthcare), then labeled with CD33 microbeads (Miltenyi Biotec, 130-045-501), and sorted with MACS (Miltenyi Biotec).

MTT assay

4T1 cells (3×10^3) starved under 1% FBS for 24 hours were plated in each well of 96-well plate and incubated overnight. The cells were then cultured in rmCCL9 containing media for 2 hours, followed by treatment of low serum (1%), glucose free conditions, or doxorubicin (LC Laboratories, gift from Dr. Beverley Mock, NCI.

Concentration: 1, 2, 10, 100, 250 nM). MTT assays were performed 24 hours later following the protocol below (Sigma, M5655).

1. Dissolve MTT to 5 µg/µl in diH₂O or PBS. This can be stored in 4°C for few months.
2. Add MTT dilute 5 µl per 96-well plate well or 50 µl per 12-well plate well.
3. Incubate in 37°C for 1-3 hours.
4. Aspirate off media.
5. Add DMSO 40 µl per 96-well plate well or 400 µl per 12-well plate well.
6. Read absorbance at 570 nm wave length.

Western blotting

Protein extraction buffer was diluted with diH₂O from 2× lysis buffer (RayBiotech, 0103004), with addition of proteinase/phosphatase inhibitor (Pierce Biotechnology, 87785 and 78420) and PMSF (1:1000 dilution from 100mM). Protein concentrations were measured using DC Protein Assay Kit (Biorad, 500-0111). Supernatants from culture were concentrated using Amicon Centrifuge Filter Unit (Millipore, UFC800324). For the TGF-β signaling pathway study, sorted myeloid cells were directly subjected to protein extraction (basal state) or starved under 1% FBS for 5 hours before treated with TGF-β1 for 1 hour (Cell Signaling, 5231LC, 5 ng/ml). Primary antibodies included TβRII (11888), P-p38 (9211), p38 (9212), P-SMAD2 (3101), SMAD2 (3103), PARP (9532), caspase 3 (9665), P-AKT (4058), AKT (9272), and AIF (4642). All antibodies above were purchased from Cell Signaling and used with a 1:1000 dilution. BCL-2 (Santa Cruz,

sc-7382, 1:500 dilution), CCL9 (Peprotech, 250-12, 0.15 µg/ml), and β-actin (Sigma, A1978, 1:5,000 dilution) were also used for primary antibody incubation at 4°C overnight. Anti-mouse/rabbit secondary antibodies (Bio-Rad, 170-6516 and 170-6515, 1:3000 dilution) were incubated for 1 hour at room temperature. Whole protein staining was performed using MemCode Reversible Protein Stain Kit (Thermo Scientific, 24580).

Cytokine antibody array and ELISA of CCL9 or CCL23

For the cytokine antibody array, the culture supernatant was collected and processed per the manufacturer's protocol (Raybiotech, AAM-CYT-1000). The expression levels of cytokines/chemokines were measured by dot density using the ImageJ software, which was then normalized to control dot density and presented as relative expression levels. For CCL9 and CCL23 ELISA, the cell culture supernatants or protein extractions from cells/mouse organs were collected and processed per the manufacturer's protocol (R&D, DY463 and DY131).

RT-qPCR

Total RNA was extracted using an RNeasy Mini Kit (Qiagen, 74104) and cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystem, 4368814). Relative gene expression was determined using SYBR Green PCR Master Mix (Applied Biosystem, 4309155). The comparative threshold cycle method was used to calculate gene expression and normalized to GAPDH as a reference gene.

RT-qPCR primers were as follows.

CCL9 forward: ATGAAGCCTTTTCATACTGCCCTC

CCL9 reverse: TTATTGTTTGTAGGTCCGTGGTTG

CCR1 forward: GTGTTTCATCATTGGAGTGGTGG

CCR1 reverse: GGTTGAACAGGTAGATGCTGGTC

GAPDH forward: AATGTGTCCGTCGTGGATCTGA

GAPDH reverse: GATGCCTGCTTCACCACCTTCT

Plasmid constructions

FUGW plasmid (Gift from Dr. Zheng-Gen Jin, University of Rochester) was used for generating CCL9/CCR1 overexpression vectors. CCL9 and CCR1 cDNA were ordered from Open Biosystem (EMM1002-99608821 and MMM1013-65208). PCR amplified mCCL9 sequence was inserted into BamHI and EcoRI digested FUGW. mCCR1 was firstly inserted into BamHI and XhoI digested PCDNA3.1-V5-B for V5 tag. mCCR1-V5 was then inserted into FUGW digested with BamHI and BsrGI.

PLKO.1 plasmid (Gift from Dr. Jing Huang, NCI) was used for CCL9 and CCR1 knockdown vectors. ShRNA sequences targeting CCL9 or CCR1 were obtained from Sigma MISSION RNAi (Table below) and were synthesized by Invitrogen. These oligos were aligned and inserted into PLKO.1 vector that was digested with AgeI and EcoRI. Sequencing around the inserts was performed to verify the vectors. Restriction enzymes

BamHI (R0136), EcoRI (R0101), XhoI (R0146), BsrGI (R0575), and AgeI (R0552) were all purchased from New England Biolabs.

CCL9 and CCR1 shRNA oligo sequences

CCL9

Sh1-1	CCGGGCCCTTTAGTTAGTAGTATTTCTCGAGAAATACTACTAACTAAAGGGCTTTTTG
Sh1-2	AATTCAAAAAGCCCTTTAGTTAGTAGTATTTCTCGAGAAATACTACTAACTAAAGGGC
Sh2-1	CCGGCCTGTCCTATAACTCACGGATCTCGAGATCCGTGAGTTATAGGACAGGTTTTG
Sh2-2	AATTCAAAAACCTGTCCTATAACTCACGGATCTCGAGATCCGTGAGTTATAGGACAGG
Sh3-1	CCGGCGGAGAGTTCAGAGATGCATTCTCGAGAATGCATCTCTGAACTCTCCGTTTTG
Sh3-2	AATTCAAAAACGGAGAGTTCAGAGATGCATTCTCGAGAATGCATCTCTGAACTCTCCG
Sh4-1	CCGGGAAATGTTTCACATGGGCTTTCTCGAGAAAGCCCATGTGAAACATTTCTTTTTG
Sh4-2	AATTCAAAAAGAAATGTTTCACATGGGCTTTCTCGAGAAAGCCCATGTGAAACATTTCT
Sh5-1	CCGGTCACACATGCAACAGAGACAACCTCGAGTTGTCTCTGTTGCATGTGTGATTTTTG
Sh5-2	AATTCAAAAATCACACATGCAACAGAGACAACCTCGAGTTGTCTCTGTTGCATGTGTGA

CCR1

Sh1-1	CCGGACCAACTAGAAAGGACCAGTTCTCGAGAACTGGTCCTTTCTAGTTGGTTTTTT
Sh1-2	AATTAAAAAACCAACTAGAAAGGACCAGTTCTCGAGAACTGGTCCTTTCTAGTTGGT
Sh2-1	CCGGCCCTGTATTCTCTAGTGTTTACTCGAGTAAACACTAGAGAATACAGGGTTTTT
Sh2-2	AATTAAAAACCCTGTATTCTCTAGTGTTTACTCGAGTAAACACTAGAGAATACAGGG
Sh3-1	CCGGCCTGTTGACGATTGACAGATACTCGAGTATCTGTCAATCGTCAACAGGTTTTT
Sh3-2	AATTAAAAACCTGTTGACGATTGACAGATACTCGAGTATCTGTCAATCGTCAACAGG
Sh4-1	CCGGCCCTACAATCTGAGTGTATTTCTCGAGAAATACACTCAGATTGTAGGGTTTTT
Sh4-2	AATTAAAAACCCTACAATCTGAGTGTATTTCTCGAGAAATACACTCAGATTGTAGGG
Sh5-1	CCGGGCCTCTGTTAGTCATGATAATCTCGAGATTATCATGACTAACAGAGGCTTTTT
Sh5-2	AATTAAAAAGCCTCTGTTAGTCATGATAATCTCGAGATTATCATGACTAACAGAGGC

Transfection and electroporation

Tumor cells (67NR, B16F1) and RAW264.7 cells were transfected with LipofectaminLTX per the manufacturer's protocol (Invitrogen, 15338-500). Gr-1+CD11b+ cells sorted from spleens of tumor-bearing wild type, $Tgfb\beta 2^{MyeKO}$, and $Tgfb\beta 2^{Flox}$ mice were transfected using an Amaxa mouse macrophage nucleofector kit per the manufacturer's instructions (Lonza, VPA1009). In brief, $2-3 \times 10^6$ Gr-1+CD11b+ cells were transfected with 5-15 μ g vector, using Nucleofector Device (Lonza) with program Y-001.

Statistical analysis

Graphpad Prism v5.04 was used for graphs and for statistical analyses. Data were presented as mean \pm standard error (SE). All data were analyzed using the student's t-test for comparison of two groups or one-way ANOVA for three groups or more. Differences were considered statistically significant when the p-value < 0.05 .

CHAPTER III

CCL9 AND ITS RECEPTOR CCR1

Introduction

Chemokine (C-C motif) ligand 9 (CCL9) is a small inflammatory cytokine (12kD). It is also known as macrophage inflammatory protein-1 gamma (MIP-1 γ), protein-related protein-2 (MRP-2), and CCF18.

Macrophages and myeloid cell lines constitutively express CCL9. This was reported in 1995 when CCL9 was firstly discovered using three independent approaches. (1) PCR CCL9 cDNA from macrophage cell lines with a degenerate PCR primer set, which was designed from the conserved amino acid sequence among C-C chemokines (Youn et al., 1995). (2) cDNA library subtraction between two cDNA libraries derived from EGF-stimulated and non-stimulated EGFR transfected B cell lines Ba/F3 (Hara et al., 1995). (3) cDNA library screening of RAW264.7 cells (Poltorak et al., 1995). In addition, CCL9 is expressed by osteoclasts (Lean et al., 2002) and microglia (Ravindran et al., 2010).

CCR1 (CC chemokine receptor type 1) is the only reported CCL9 receptor. CCR1 is a seven transmembrane protein that interacts with multiple other ligands, including CCL3/MIP-1 α , CCL5/RANTES (regulated on activation, normal T cell expressed and secreted), CCL7, and CCL23 (Neote et al., 1993).

CCR1 is expressed in both host and cancer cells. Immune cells including monocytes, lymphocytes, and dendritic cells express CCR1 (Ogata et al., 1999; Su et al., 1997).

Cancer cells including melanoma, breast, and lung cancer cells also express CCR1 (Soria et al., 2008; Vallet and Anderson, 2011; Wang et al., 2009). CCR1 knockdown decreases tumor cell invasion (Wang et al., 2009; Wu et al., 2007). Furthermore, CCR1 positively correlates with the expression levels of stem cell markers SOX2, OCT4, and NANOG (Kauts et al., 2013). Overexpression of CCR1 increases the cell viability of mesenchymal stem cells (MSCs) (Huang et al., 2010).

CCL9/CCR1 has been shown to be important for the recruitment of dendritic and myeloid progenitors, as well as immature myeloid cells (iMCs) (Kitamura et al., 2007; Zhao et al., 2003). In particular, recruited iMCs to the intestinal tumors enhance tumor invasion and metastasis (Kitamura et al., 2007). CCL9 knockdown or CCR1 blockade significantly decreases tumor progression *in vivo* by reducing iMC recruitment (Kitamura et al., 2010). The CCL9/CCR1 axis is also the major chemokine/receptor axis expressed by osteoclasts for the regulation of bone resorption (Lean et al., 2002). Additionally, CCL9 functions as a cell survival factor for osteoclasts (Okamatsu et al., 2004). It remains unknown whether CCL9 produced by myeloid cells signals through CCR1 on tumor cells to function as a tumor cell survival factor.

Results

CCL9 is significantly produced in the supernatant of Gr-1+CD11b+ and tumor cell co-culture

To address the question whether tumor-educated myeloid cells secrete factors that directly promote tumor metastasis at the metastatic site, we co-cultured Gr-1+CD11b+ cells with 4T1 mammary tumor cells and performed a cytokine protein array analysis on culture supernatants. We sorted Gr-1+CD11b+ cells from spleens of 4T1 tumor-bearing mice since they showed a very similar cytokine profile to that of Gr-1+CD11b+ cells derived from the lungs of tumor-bearing mice (Fig. 4). Using splenic Gr-1+CD11b+ cells was technically more feasible, as enlarged spleens contained a large population of these cells in mice bearing 4T1 tumors (Yang et al., 2004).

Interestingly, we found that CCL9 was significantly induced in the myeloid-tumor co-culture supernatant but not in the supernatant of tumor or myeloid cells cultured alone (Fig. 5a and b, red boxes). In contrast, other CC family members, including CCL12, CCL3, and CCL19, exhibited low expression and did not change significantly in co-culture. CCL2, CCL20, and CXCL1 were predominantly produced by tumor cells (Fig. 5a and b, yellow, cyan, and purple boxes, orderly), in agreement with previous publications that tumor cells produce and secrete these cytokines to recruit inflammatory monocytes (Qian et al., 2011), immature dendritic cells (Bell et al., 1999), and Gr-1+CD11b+ cells (Acharyya et al., 2012). MMP9, a key regulator for tumor vascular remodeling (Bergers et al., 2000), was also expressed by these myeloid cells (Fig. 5a and

b, blue boxes), consistent with our earlier studies (Yang et al., 2004). This observation of CCL9 expression in myeloid-tumor co-culture was confirmed using CCL9 ELISA (Fig. 6a).

To determine whether CCL9 overexpression in the co-culture supernatant was cell line specific, we looked at different cell lines, including B16BL6 melanoma in C57BL/6 background and non-metastatic 67NR mammary gland tumor in BALB/c background. CCL9 was also overexpressed in these co-culture supernatants (Fig. 6b and c). We therefore focused our efforts on investigating CCL9.

CCL9 is expressed by myeloid cells

It was important to understand which cell types expressed CCL9 in order to design functional studies involving CCL9 knockdown or overexpression. To address this question, we separated adherent tumor cells and non-adherent myeloid cells for both RT-qPCR and CCL9 ELISA. Both RNA (Fig. 7a) and protein levels (Fig. 7b and c) indicated that only myeloid cells expressed CCL9, confirming that tumor cells did not produce CCL9 in our system (Fig. 5).

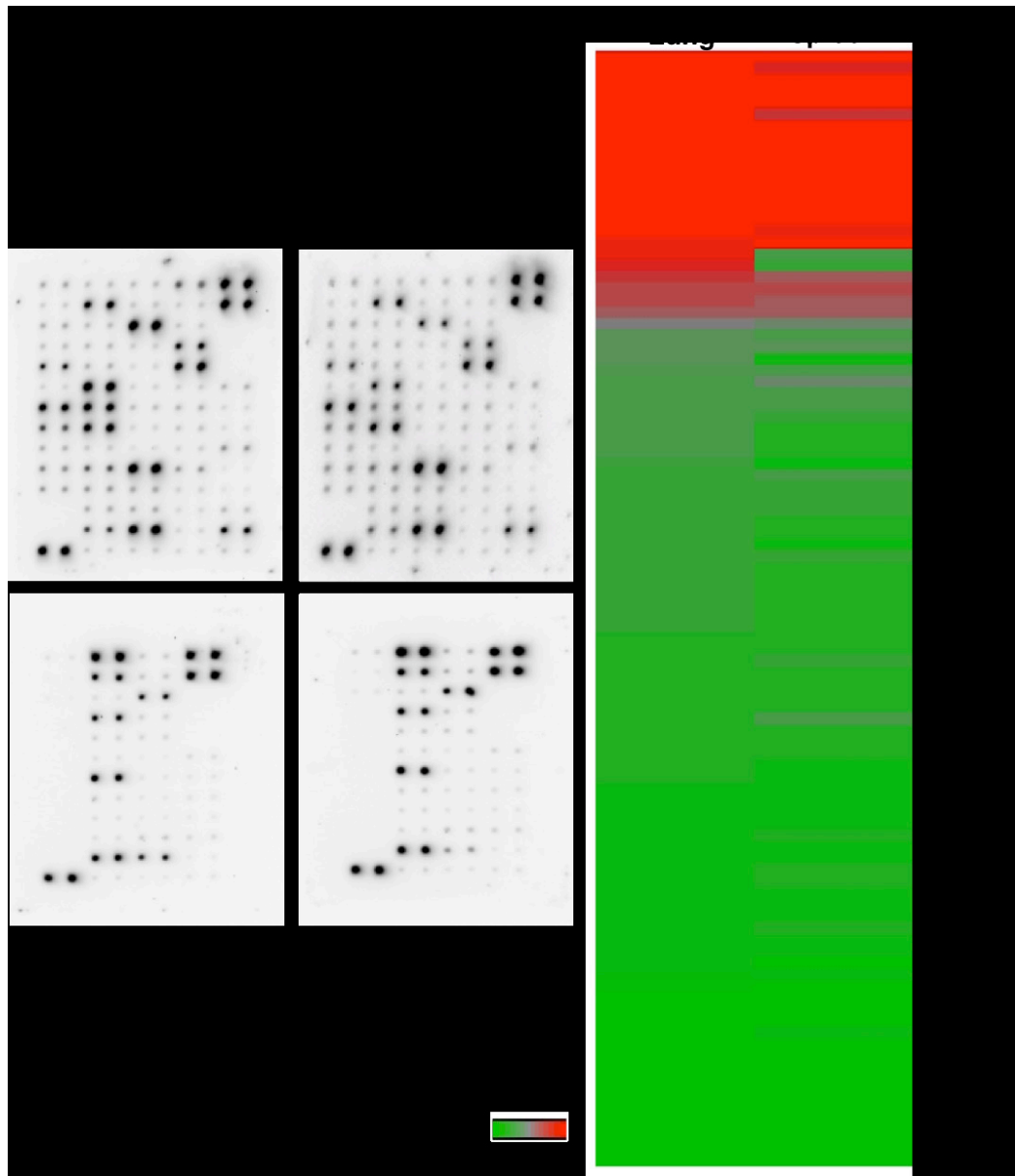


Figure 4. Similarity of cytokine profile between splenic Gr-1+CD11b+ cells and those derived from premetastatic lungs of tumor-bearing mice.

(a) Cytokine protein array of Gr-1+CD11b+ myeloid cells sorted from lungs (left two panels) and spleens (right two panels) of tumor-bearing mice 14 days after tumor injection. **(b)** Heat map showing semi-quantitative analysis of (a). Dot density was normalized to control dot density and data were presented as relative dot density. Pooled samples from 5 mice were used for each group. The myeloid cells were cultured in tumor-conditioned media for 24 hours, and the culture supernatant was used for array.

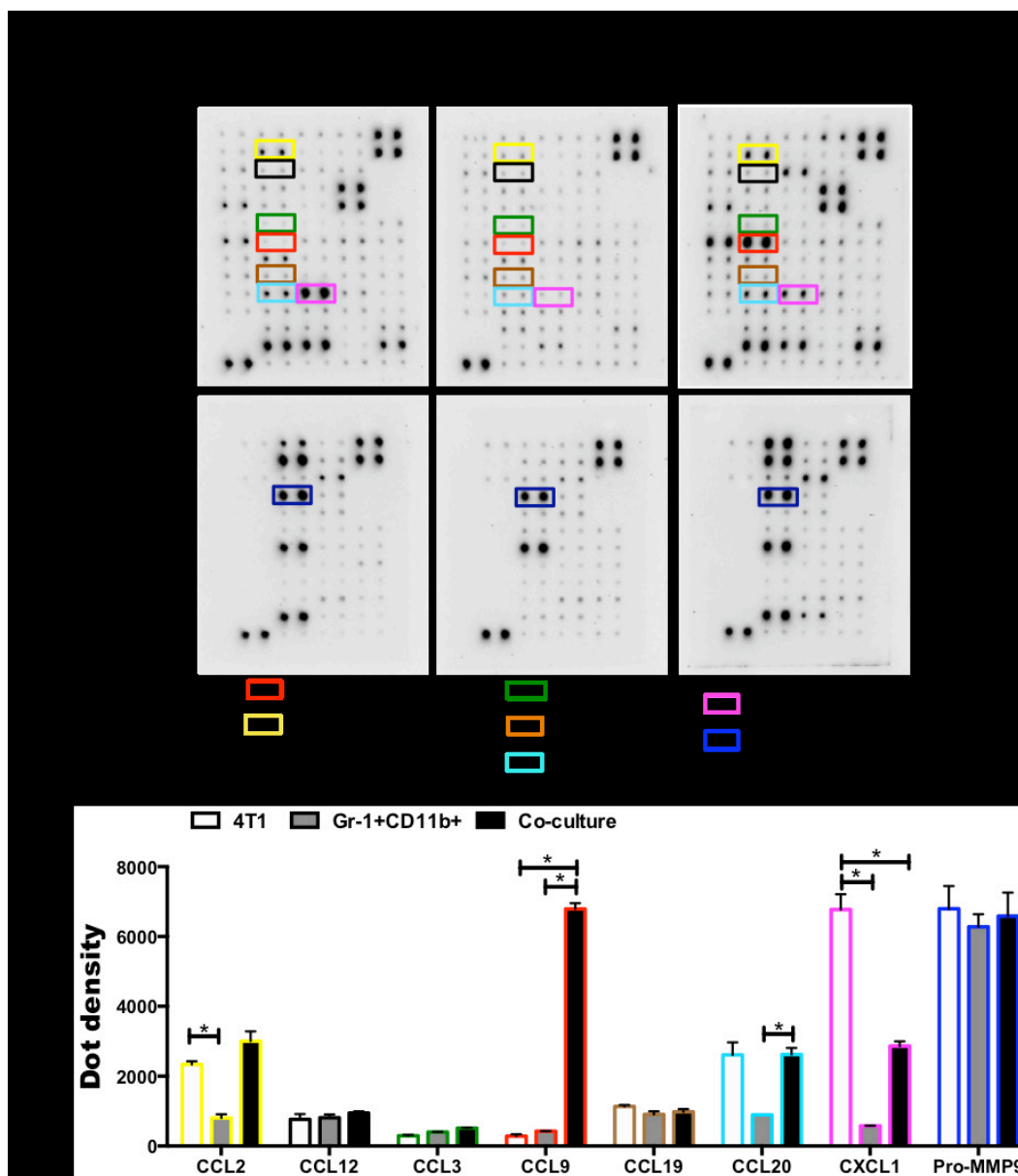


Figure 5. Elevated CCL9 secretion in the co-culture of 4T1 tumor and Gr-1+CD11b+ cells.

(a) Cytokine protein array of supernatants from 24 hours cultured 4T1 tumor, Gr-1+CD11b+ myeloid, and myeloid-tumor co-culture. Dots representing CCL9 and other CC family members were highlighted with colored boxes. One of two experiments performed was presented. **(b)** Semi-quantitative data of (a). Data presented as dot density. Pooled samples from 3 mice were used for Gr-1+CD11b+ cell sorting. *P<0.05.

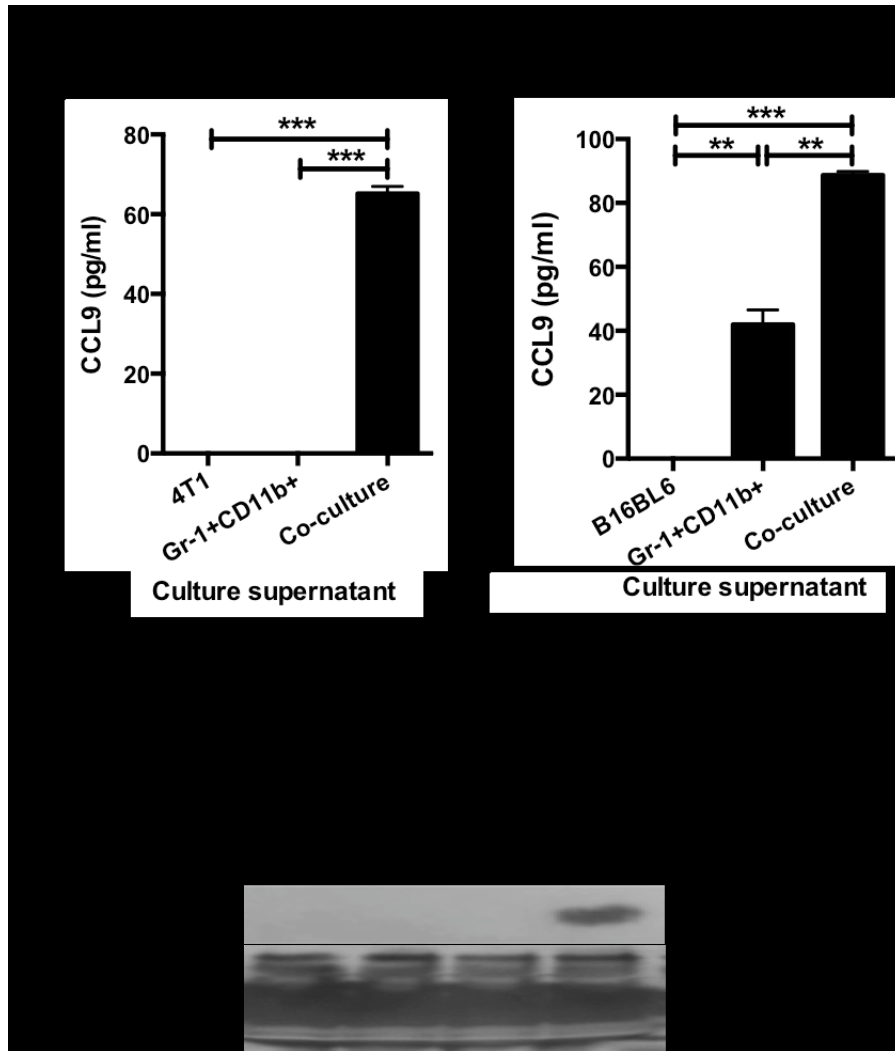


Figure 6. CCL9 secretion in other tumor models.

ELISA or Western blots detecting CCL9 expression in the supernatants of (a) 4T1, (b) B16BL6, and (c) 67NR cells, or when co-cultured with splenic Gr-1+CD11b+ cells from mice bearing tumors of the same cell line. Supernatants from tumor or Gr-1+CD11b+ cells alone served as controls. Whole protein staining on Western blot showed equal protein loading. **P<0.01, ***P<0.001.

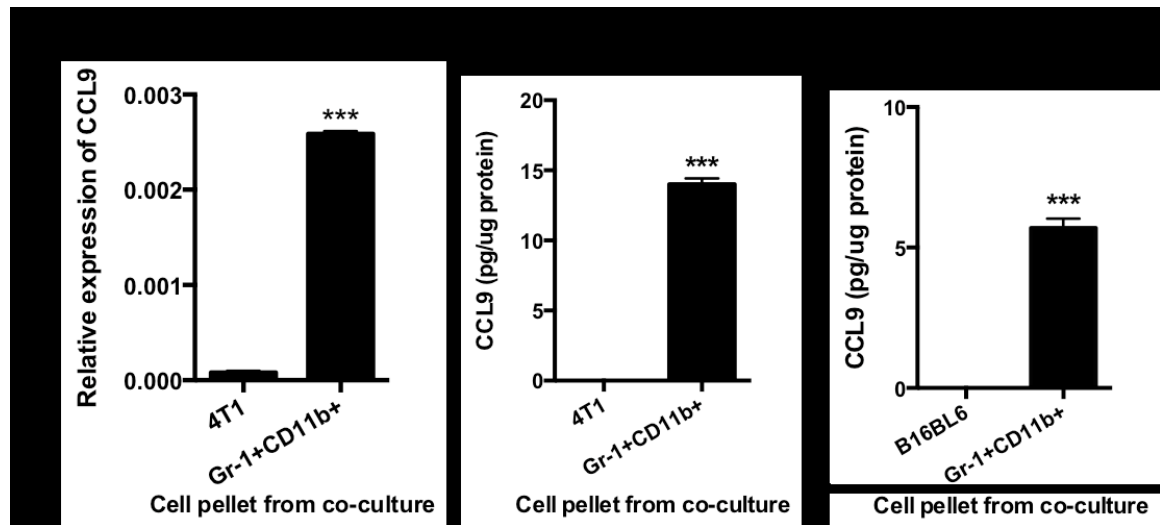


Figure 7. CCL9 was produced by myeloid cells.

(a) RT-qPCR showing CCL9 mRNA expression. ELISA showing CCL9 protein expression in the supernatant of Gr-1+CD11b+ cells co-cultured with either (b) 4T1 or (c) B16F10. Cell pellets were collected from myeloid-tumor co-culture. ***P<0.001.

CCL9 is significantly produced in the premetastatic lungs

We next tested whether CCL9 was up-regulated in the premetastatic lungs, as Gr-1+CD11b+ cells and other immature myeloid cells had been reported to be present in the lung prior to tumor cell arrival (Hiratsuka et al., 2006; Kaplan et al., 2005; Yan et al., 2010). The 4T1 and B16F10 models had been reported to have a premetastatic niche established after tumor cell inoculation, on day 14 for the 4T1 model (Kowanetz et al., 2010; Yan et al., 2010) and on day 18 for the B16 model (Kaplan et al., 2005). As expected, there was a significantly higher CCL9 expression in the premetastatic lungs compared to normal lungs in both 4T1 and B16F10 models (Fig. 8a and b). This elevated CCL9 production seen in the premetastatic lungs was not observed in other organs at the premetastatic phase of mice bearing 4T1 (Fig. 8d) and B16F10 tumors (Fig. 8e), suggesting an organ specificity for the premetastatic niche formation.

Cell-cell contact is not necessary for CCL9 expression

To ask whether direct contact between tumor cells and Gr-1+CD11b+ cells was necessary for CCL9 expression, we cultured Gr-1+CD11b+ cells in tumor-conditioned media or in direct contact with tumor cells, and measured CCL9 concentrations in the supernatant. Interestingly, Gr-1+CD11b+ cells cultured in tumor-conditioned media produced high levels of CCL9 (Fig. 8c). However, CCL9 was produced at a higher level in the supernatant where Gr-1+CD11b+ cells were co-cultured with tumor cells (Fig. 8c), suggesting that although cell-cell contact was not necessary, proximity enhanced CCL9

expression.

Only subset Ly6G+CD11b+ cells express CCL9 when co-cultured with tumor cells

To investigate which cell subset(s) were responsible for CCL9 production, we FACS sorted Ly6G+CD11b+ granulocytes, Ly6C+CD11b+ monocytes, and F4/80+CD11b+ macrophages (Fig. 9a). These myeloid subsets were then co-cultured with tumor cells, and CCL9 production was measured using ELISA. Sorted Ly6G+CD11b+ cells produced lower levels of CCL9 than either Ly6C+CD11b+ or F4/80+CD11b+ subsets. However, CCL9 production was elevated 20-fold in Ly6G+CD11b+ cells, while remaining mostly unchanged in the Ly6C+CD11b+ and F4/80+CD11b+ subsets upon co-culture with tumor cells (Fig. 9b). In addition, there were significantly more Ly6G+CD11b+ cells in the premetastatic lung (day 14 after 4T1 tumor injection), while the populations of other cell types were unchanged (Fig. 9c). Taking cell number into consideration, CCL9 increase was far greater in the Ly6G+CD11b+ cells (Fig. 9d).

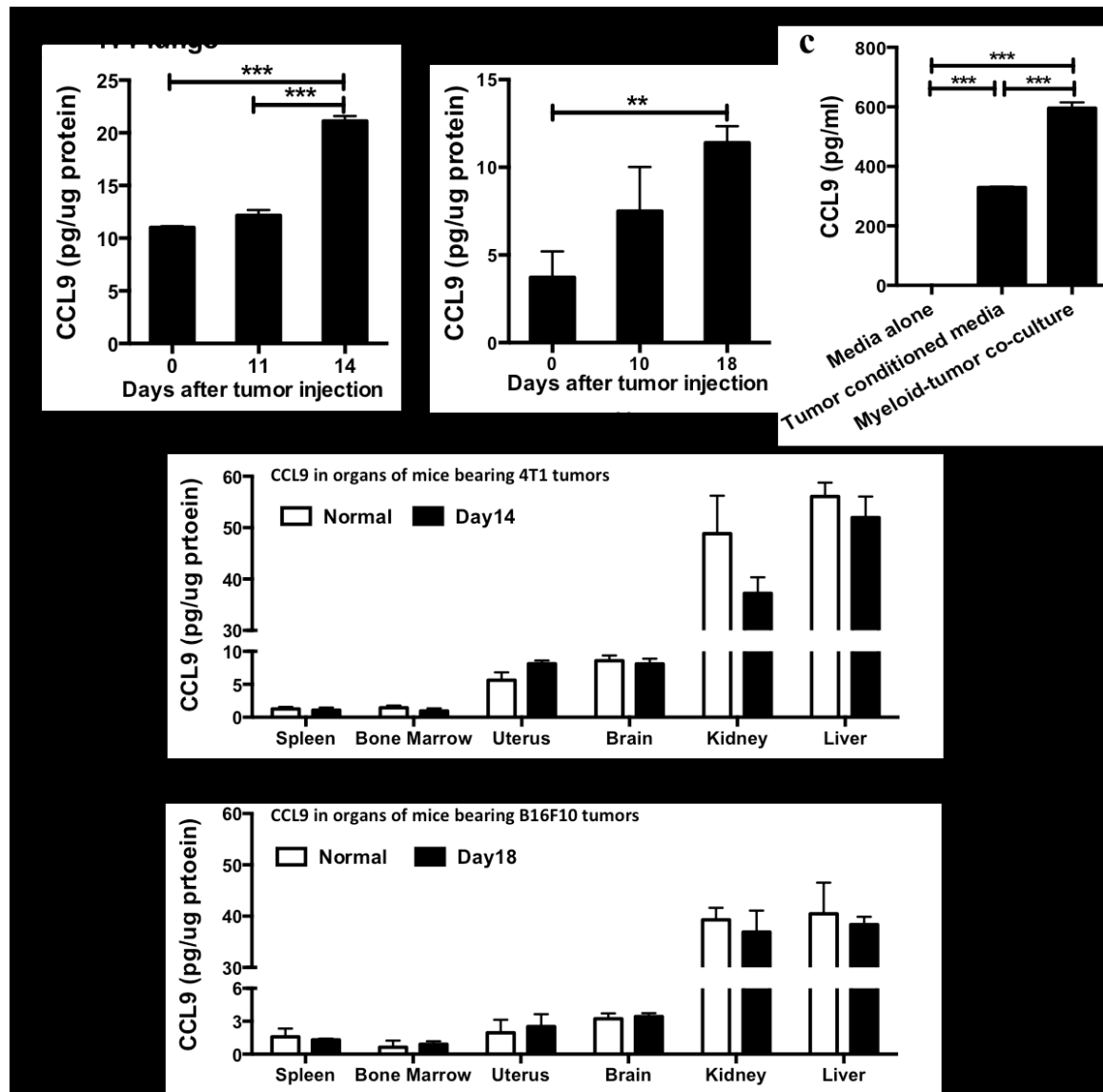


Figure 8. CCL9 was significantly up-regulated in the premetastatic lungs.

ELISA detecting CCL9 expression in the normal and the premetastatic lungs of (a) 4T1 and (b) B16BL6 tumor-bearing mice. CCL9 expression was also measured in other organs at 14 days (d) and 18 days (e) after tumor inoculation. n=3 mice for each group. Equal amount of protein extracts were loaded onto the plate and results were presented as pg CCL9/μg protein. (c) ELISA assay detecting CCL9 secretion in the supernatant of Gr-1+CD11b+ cells when cultured with 4T1 tumor-conditioned media or with tumor cells. **P<0.01, ***P<0.001.

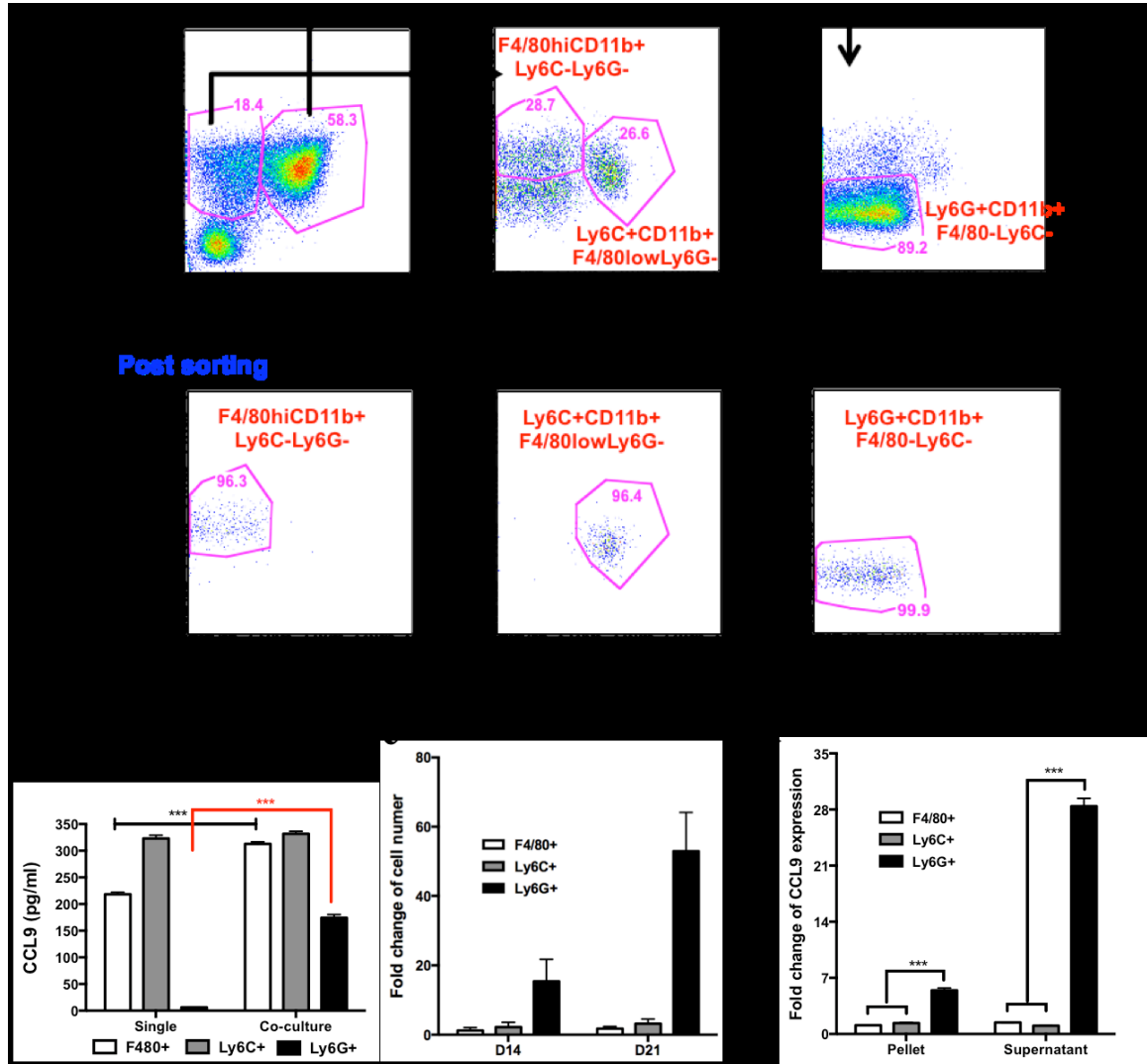


Figure 9. Myeloid cell subsets in CCL9 production.

(a) Upper panel: sorting strategy of Ly6G⁺, Ly6C⁺, and F4/80⁺ myeloid cell subsets. Lower panel: post-sorting flow cytometry analysis. **(b)** ELISA showing CCL9 expression in subsets upon co-culture with tumor cells. **(c)** Fold increase in myeloid cell subsets in the premetastatic lung (Day 14) and later tumor-bearing stage (Day 21). Fold change in cell number was calculated using normal mice as base line. **(d)** Fold change in CCL9 expression from myeloid-tumor co-culture supernatant with the consideration of the fold increase in cell number. ***P<0.001

CCR1 is expressed by tumor cells

Since CCR1 is the sole receptor mediating CCL9 signaling (Kitamura et al., 2010; Kitamura et al., 2007; Lean et al., 2002; Zhao et al., 2003), we next examined the correlation of CCR1 expression in tumor cells with cancer metastasis. We used 4T1 mammary tumor and B16 melanoma models comprised of cell lines with varying degrees of metastatic capability. The CCR1 expression level correlated with the degree of malignancy across both cell line panels (Fig. 10a and b). The low metastatic 67NR and 168FARN cells showed minimum expression of CCR1 mRNA, whereas the highly metastatic 4TO7 and 4T1 cells showed approximately 10-fold higher expression (Fig. 10a). Likewise, the less metastatic B16F1 cells had a lower level of CCR1 mRNA expression compared to highly metastatic B16F10 cells (Fig. 10b). Flow cytometry analysis revealed approximately 8% of 4T1 cells express CCR1, which was significantly higher than that of 67NR, 168FARN, and 4TO7 cells (Fig. 10c and d). Interestingly, tumor cells from metastatic lung nodules showed higher CCR1 expression than those from either the primary tumor tissues or cells in culture (Fig. 10e).

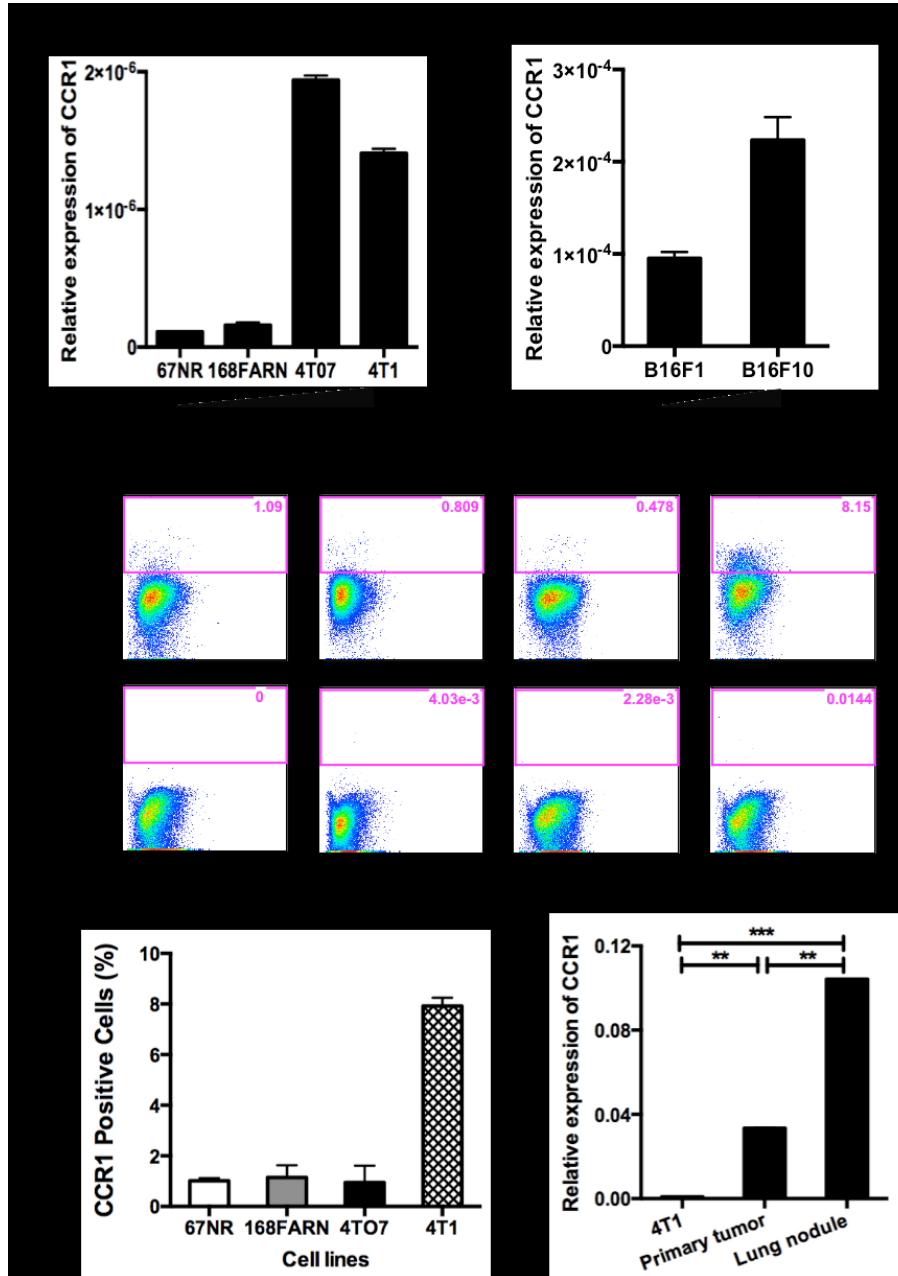


Figure 10. CCR1 expression in tumor cells correlated with metastatic capability. RT-qPCR detecting CCR1 expression in (a) mammary gland tumor series and (b) melanoma cell line series (c,d) Flow cytometry detecting CCR1+ tumor cells in mammary gland tumor series. (e) RT-qPCR detecting CCR1 expression in tumor cell lines, primary tumors, and tumor nodules in the lung. **P<0.01, ***P<0.001.

Correlation of human CCL23/CCR1 and metastasis free survival

To establish the clinical significance of our study, we next looked into the correlation between CCL23 expression levels and metastasis. CCL23 had been identified as the human orthologue for mouse CCL9 (Kitamura et al., 2007; Kowanetz et al., 2010). They shared 92-99% sequence identity when examined by the NCBI web tool BLAST (Fig. 11a) and clustered together by Treefam analysis (Fig. 11b). They were also predicted orthologues by MetaPhOrs online tool (Fig. 11c) and showed similar chemoattractant functionality (Kitamura et al., 2007; Patel et al., 1997; Zhao et al., 2003). In addition, human CCL23 and mouse CCL9 were two of the four NC6 subfamily members in C-C chemokines (Berahovich et al., 2005).

Using a publicly available dataset (Rotunno et al., 2011), we examined the expression level of CCL23 in human peripheral blood mononuclear cells (PBMCs) in a cohort of lung cancer patients that contains 48 patients in stage I and II, 30 patients in stage III and IV, and 80 healthy individuals. CCL23 was the only up-regulated gene in the later tumor stage among all 19 CC cytokines available in this dataset (Fig. 12a). Of the remaining cytokines, CCL4 was the only decreased while all other CC cytokines showed no change (data not shown). In addition, meta-analysis of 1577 breast cancer patients in the GOBO breast cancer database (Ringner et al., 2011) revealed that patients with low expression of CCL23/CCR1 had a better survival outcome among 166 PAM50 HER2+ patients (Fig. 12b), which was not observed in the HER2- patients (data not shown). PAM50 (Prediction Analysis of Microarray using 50-gene classifier) is a 50-gene

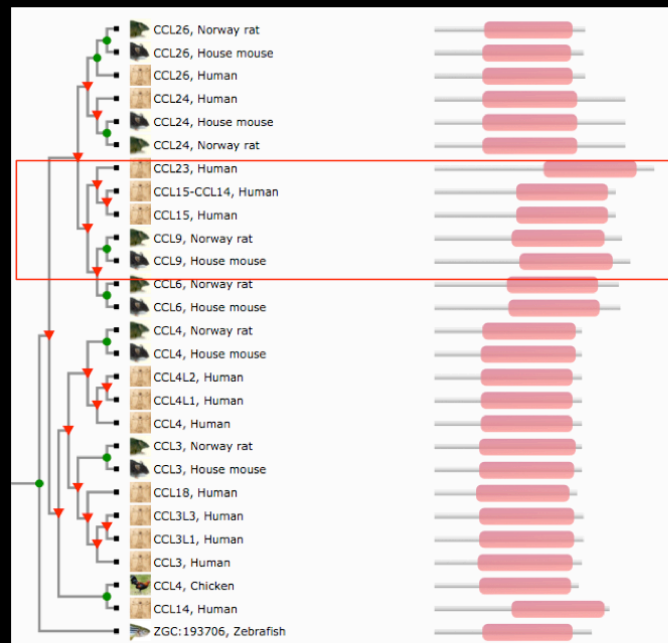
signature that adds further prognostic and predictive information to standard parameters for breast cancer patients (Parker et al., 2009). In addition, analysis using the Oncomine database (GSE20685) showed a negative correlation of CCL23 and CCR1 expression levels with metastatic status three years after breast cancer diagnosis (Fig. 12c, heat map), or with a decreased metastasis free survival (Fig. 12c, lower panels).

CCL23 expression in human myeloid cells

To examine CCL23 induction in human myeloid cells under tumor conditions, we sorted human CD33⁺ myeloid cells from the peripheral blood mononuclear cells (PBMCs) of healthy blood donors and co-cultured them with human breast cancer cell lines MDA-MB-231/MDA-MD435, lung cancer cell line H460, and melanoma cell lines A375S2/SK-MEL-28. Culture supernatants from CD33⁺ myeloid cells alone, tumor cells alone, and the myeloid-tumor co-culture were examined by CCL23 ELISA. Consistent with the mouse findings, CCL23 was significantly induced in myeloid-tumor co-culture supernatants but not in supernatants from tumor cells or myeloid cells cultured alone (Fig. 13a), and was mainly produced by myeloid cells (Fig. 13b). This observation was made with separation of adherent tumor cells from non-adherent myeloid cells for protein extraction and CCL23 ELISA (Fig. 13b). Lastly, MDA-MB-231 conditioned media induced CCL23 production (Fig. 13c). Yet, CCL23 was produced at a higher level when CD33⁺ myeloid cells were co-cultured with tumor cells, suggesting that cell-cell proximity enhanced CCL9 expression, paralleling our results in mice. Together, our data

support that CCL23 was induced in myeloid cells and correlates with human cancer metastasis.

NP_665905.1	C-C motif chemokine 23 isoform Ckbeta8 precursor [Homo sapiens]	99
NP_005055.2	C-C motif chemokine 23 isoform Ckbeta8-1 precursor [Homo sapiens]	92.4



Target species	M. musculus co-orthologs (CS)	Target orthologs	CS	Evidence level	Trees	PhylomeDB CS / EL	Ens	Egg	Ort	COG	FO	TF
Homo sapiens	2 co-orthologs	Q16663	1.000	3	22	1.000 / 1				CCL15		
	2 co-orthologs	Phy000875R	1.000	5	20	1.000 / 1				CCL23		

Figure 11. Human CCL23 was a homologue of mouse CCL9.

(a) Similarity between mouse CCL9 and human cytokines. (b) Family tree identified by the online tool Treefam. (c) Human CCL23 (Phy000875R) was identified as one of the two orthologues for mouse CCL9. Data obtained from the online tool mrtaPhOrs.

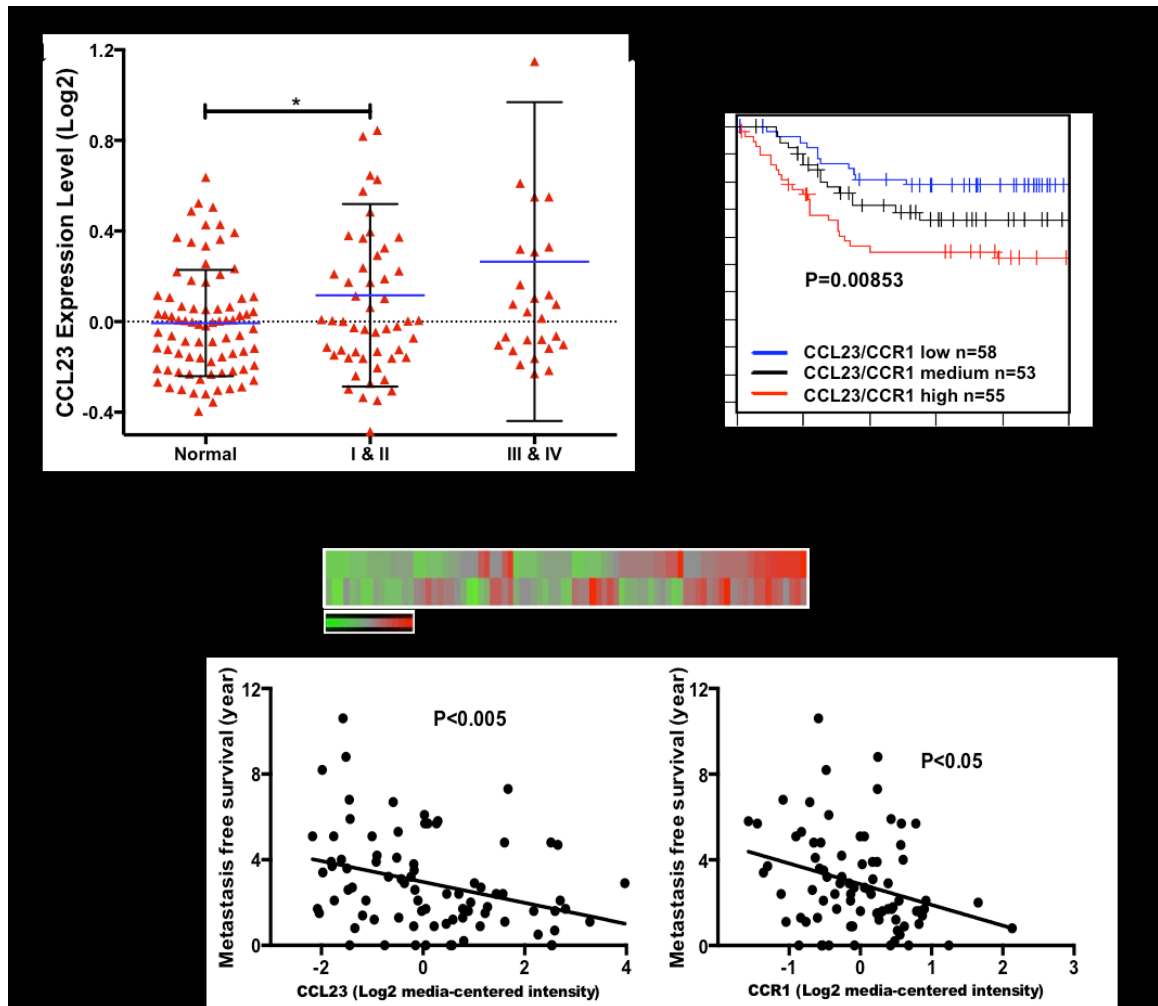


Figure 12. CCL23/CCR1 correlated with metastatic status and survival of cancer patients.

(a) CCL23 expression levels in PBMCs of a cohort of lung cancer patients and healthy donors, comprising 80 healthy donors, 48 patients in stage I and II, and 30 patients in stage III and IV. (b) Kaplan–Meier survival curve of CCL9/CCR1 expression in 166 PAM50 HER2+ breast cancer patients in GOBO database. (c) Oncomine database (GSE20685) showing a correlation of CCL23 or CCR1 expression levels with metastatic status 3 years after diagnosis (heat map) or with a decreased overall metastasis free survival (lower panels). *P<0.05.

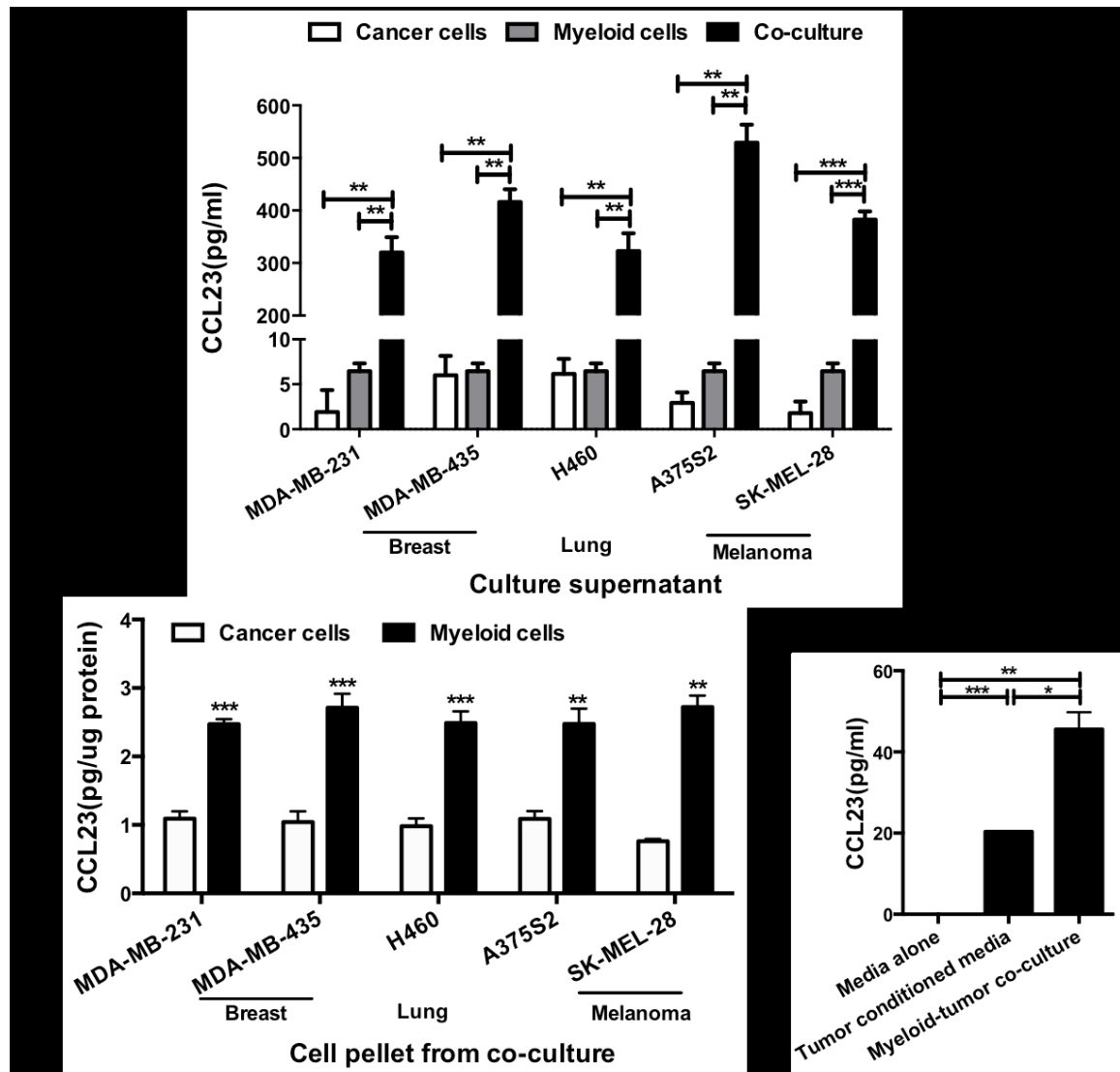


Figure 13. Human myeloid cells produced and secreted CCL23.

(a) CCL23 ELISA of co-culture supernatant. Co-culture of sorted human CD33+ PBMCs and indicated human breast, lung, or melanoma cancer cell lines. Culture supernatants from CD33+ PBMCs alone, tumor cells alone, or co-culture were also tested. **(b)** CCL23 ELISA of protein samples from adherent tumor cells or non-adherent CD33+ PBMCs after separation from co-culture. **(c)** CCL23 ELISA of sorted human CD33+ PBMCs cultured in MDA-MB-231 tumor-conditioned media or in co-culture with MDA-MB-231 cells, with triplicates of samples. CD33+ myeloid cells were sorted and pooled from healthy donors, with triplicates per treatment group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Discussion

Similarity of cytokine profile between splenic Gr-1+CD11b+ cells and those derived from the premetastatic lungs of tumor-bearing mice

Our study was interested in the tumor-promoting function of Gr-1+CD11b+ cells in the premetastatic lung. At this stage, infiltrated Gr-1+CD11b+ cells were induced by tumor secreting factors in the microenvironment to execute tumor-promoting function. We sorted out both splenic and the premetastatic lung derived Gr-1+CD11b+ cells, and they showed a very similar cytokine expression profile when cultured in tumor-conditioned media (Fig. 4), indicating that we could use splenic Gr-1+CD11b+ cells as a substitute for those from the premetastatic lung.

This substitution allowed us to overcome the challenge of isolating the small Gr-1+CD11b+ cell population from the premetastatic lung. Using 4T1 BALB/c mammary gland tumor model as one example, we could only obtain $0.5-1 \times 10^6$ Gr-1+CD11b+ cells per premetastatic lung, due to small starting cell population and cell loss/death during sorting procedures. On the other hand, we could easily harvest 2×10^8 Gr-1+CD11b+ cells from spleens of tumor-bearing mice, due to spleen enlargement and high proportion of Gr-1+CD11b+ cells. At the meantime, we were aware the possible pitfall using splenic cells, and testing key experiments with premetastatic lung derived Gr-1+CD11b+ cells would answer this question.

Direct contact is not required, but proximity promotes CCL9 expression

Our previous study showed that Gr-1⁺CD11b⁺ cells promoted lung metastasis by building up a proliferative, inflammatory, and tumor-favorable premetastatic lung microenvironment (Yan et al., 2010). However, how the secreted chemokines of premetastatic tissue differ from normal lung tissue was not clear. In this study, we found that Gr-1⁺CD11b⁺ cells only expressed and secreted CCL9 when interacting with tumor cells. Out of the near 100 cytokines that we tested, only CCL9 was induced in the myeloid-tumor co-culture supernatant. The level of CCL9 expression in co-culture was about 25-fold higher than in single cultures (Fig. 5), suggesting that CCL9 was likely to be one of the most important factors secreted by Gr-1⁺CD11b⁺ cells to promote tumor cell metastasis.

Interestingly, tumor-conditioned media stimulated CCL9 expression (Fig. 8). This result suggested that primary tumor cells might secrete signaling molecules to promote CCL9 expression. These signaling molecules could circulate through the vasculature to reach the premetastatic organ before tumor cell arrival. Tumor-conditioned media also stimulated human orthologue CCL23 production and secretion by human CD33⁺ myeloid cells, indicating that our mouse study could serve as a model to understand the biology of human premetastatic organs, as currently direct testing of the human premetastatic lung is currently difficult.

CCL9/CCR1 expression in multiple cancer models

The CCL9/CCR1 mechanism of promoting cancer metastasis could be applied to multiple cancer models. In addition to the 4T1 BALB/c model that we used for our initial cytokine screening (Fig. 5), we examined other tumor cell lines of different mouse backgrounds. These included B16BL6 in C57BL/6 background and 67NR in BALB/c background (Fig. 6). More importantly, we found a negative correlation of metastatic status and patient survival with CCL23 expression in PBMCs, which included a high number of immature myeloid cells when under tumor conditions (Fig. 12). The same correlation was shown in the examinations of CCL9/CCR1 expression in the primary tumors (Fig. 12).

Different CCL9 expression in myeloid cells when cultured alone may be due to different subset compositions

Gr-1+CD11b+ cells include Ly6G+, Ly6C+, and F4/80 three subsets. We observed that only Ly6G+ cells significantly increased CCL9 production when stimulated by tumor cells. The other two subsets, although producing CCL9 without tumor cell stimulation, did not further increase the CCL9 secretion (Fig. 9). Since Ly6G+ was the major cell subset in the premetastatic lung, the effect of Ly6G+ cells in terms of CCL9 expression was far greater than the Ly6C+ or F4/80+ subsets (Fig. 9). As a result, Ly6G+ should be the targeted cell type for the future studies of CCL9 neutralization or knockdown.

Our initial cytokine protein screen in the 4T1 tumor model showed that CCL9 was not secreted by Gr-1+CD11b+ cells when cultured alone (Fig. 5). We further confirmed this finding by using both ELISA and Western blots (Fig. 6). However, Gr-1+CD11b+ cells secreted some CCL9 when sorted from spleens of B16BL6 tumor-bearing C57BL/6 mice and cultured alone (Fig. 6). This was likely due to the differences in subset compositions between different animal backgrounds. As shown in Fig. 9 of single cultures, both Ly6C+ and F4/80 subsets expressed CCL9, whereas Ly6G+ exhibited almost no CCL9 expression.

Gr-1+CD11b+ cells in 4T1 and 67NR models were from BALB/c background, and Gr-1+CD11b+ cells in B16BL6 models were from C57BL/6 background. Compared to BALB/c, C57BL/6 mice were known to polarize in type 1 immune responses with higher resistance to diseases (Chaudhri et al., 2004). Thus, there was a higher percentage of F4/80 myeloid cells in mice of the C57BL/6 background, which exhibit elevated CCL9 expression (Fig. 9).

CCL9 is expressed mostly in myeloid but not tumor cells, different from some previous publications

Our data were in agreement with reports that CCL9 was secreted in macrophages and myeloid cell lines including mouse 32D neutrophil, P388D1/RAW264.7 macrophage cell lines (Hara et al., 1995; Okamatsu et al., 2004). These data were different from two previous reports in which CCL9 was expressed and secreted by tumor cells (Kitamura et

al., 2007; Owens et al., 2012). However, neither paper had a clean system where tumor cells were exclusively separated. One paper performed IHC staining on colon tumor tissue, as well as RT-qPCR on laser captured tumor tissue (Kitamura et al., 2007). The other paper cultured primary mammary tumor and detected CCL9 in the culture supernatant (Owen-Schaub et al., 1998). Both papers looked at CCL9 expression in the whole tumor tissue, which was known to have a high residency of myeloid cells and other immune cells (Yang et al., 2004). This may well account for the differences in our data.

CHAPTER IV
THE PARACRINE AND AUTOCRINE EFFECT OF CCL9/CCR1:
PROMOTING THE SURVIVAL OF TUMOR AND GR-1+CD11B+ CELLS

Introduction

Tumor cell survival is the rate-limiting step in cancer metastasis. Metastasis is an inefficient process, as the majority of cancer cells die in the peripheral blood or distant hostile organs before they reach their full metastatic potential (Mehlen and Puisieux, 2006).

The host effect is important in promoting tumor cell survival. Evidence from recent years strongly suggests that host cells in the microenvironment, especially myeloid cells in the secondary organ, play a critical role in enhancing tumor cell survival and metastasis (Kang and Pantel, 2013). For example, macrophages expressing $\alpha 4$ integrin that binds to vascular cell adhesion molecule-1 (VCAM-1) expressed on tumor cells (Chen et al., 2011); Gr-1+CD11b+ cells expressing S100A8/9 that promote tumor cell survival (Acharyya et al., 2012). We have reported that Gr-1+CD11b+ cells are significantly recruited in the premetastatic lung and to modulate the microenvironment through cytokine/chemokine expressions (Yan et al., 2010). However, it is unknown whether these myeloid cells can directly support tumor cell survival in the premetastatic organs and what the molecular mechanisms may be.

Whether CCL9/CCR1 promotes tumor metastasis through enhanced tumor cell survival is unknown. CCL9 is a known cell survival factor for osteoclasts. CCL9

neutralization significantly decreases cell survival stimulated by RANKL (receptor activator of NF- κ B ligand) *in vitro* compared to the control treated group (Okamatsu et al., 2004). Additionally, CCR1 promotes MSC survival as overexpression of CCR1 significantly reduces TUNEL+ cells that are induced by serum depletion (Huang et al., 2010). CCL9/CCR1 is reported to promote metastasis through recruitment of CCR1+ myeloid cell to assist tumor cell invasion (Kitamura et al., 2007). CCL9 knockdown or CCR1 blockade significantly decreases tumor progression *in vivo* by reducing the accumulation of iMCs at the tumor invasive front (Kitamura et al., 2010). It remains unknown whether CCL9 directly signals through CCR1 and functions as a tumor cell survival factor.

In Chapter III, we discovered a significant increase of CCL9 production and secretion by the Gr-1+CD11b+ cells when co-cultured with tumor cells, and in the premetastatic lung compared to the normal lung. In this chapter, we will address the biological function of CCL9 produced by Gr-1+CD11b+ myeloid cells.

Results

CCL9/CCR1 signaling promotes tumor metastasis

Immature myeloid cells had been shown to promote cancer metastasis (Kang and Pantel, 2013; Psaila and Lyden, 2009). Consistent with this, luciferase labeled 4T1 (4T1-luc) mammary tumor cells, when co-injected with Gr-1+CD11b+ cells, showed increased metastasis. This result was shown by luciferase image quantification (Fig. 14a and b). 67NR, a relatively non-metastatic cell line derived from the same mammary tumor as 4T1, showed a 3-fold increase in metastasis when co-injected with Gr-1+CD11b+ cells by tail vein injection (Fig. 14c).

To overcome the technical difficulties of gene modification in primary Gr-1+CD11b+ cells, we firstly explored whether RAW264.7 macrophage or 32Dcl3 neutrophil cell lines could be used for stable CCL9 knockdown. Both cell lines constantly expressed CCL9 (Hara et al., 1995; Okamatsu et al., 2004) and they might produce cytokines similar to Gr-1+CD11b+ cells under tumor conditions. When cultured in 4T1 tumor-conditioned media, RAW264.7 macrophages showed a similar cytokine profile to primary Gr-1+CD11b+ cells, especially in their high level expression of CCL9, which was not seen in the 32Dcl3 neutrophil cell line (Fig. 15a and b, blue boxes). In addition, RAW264.7 cells showed a metastasis-promoting effect similar to Gr-1+CD11b+ cells when co-injected with tumor cells through the tail vein (Fig. 15c). We next decided to knockdown CCL9 in RAW264.7 cells to study the function of myeloid CCL9 in tumor metastasis.

To determine whether CCL9 is a critical mediator in the myeloid promotion of cancer metastasis, we transiently knocked down CCL9 in Gr-1+CD11b+ cells. We were able to achieve about 40% knockdown in primary Gr-1+CD11b+ cells (Fig. 16a). CCL9-deficient Gr-1+CD11b+ cells significantly decreased lung metastasis compared to the control group (Fig. 16b). This metastasis difference was not due to altered primary tumor sizes (Fig. 16c). Next, we stably knocked down CCL9 in RAW264.7 cells. We utilized two shRNAs that represented 50% and 90% knockdown (Fig. 16d). Again, 67NR tumor cells showed a decreased lung metastasis when co-injected with CCL9-deficient RAW264.7 cells compared to those co-injected with the control RAW264.7 cells (Fig. 15e and f). The degree of metastasis inhibition correlated with the level of CCL9 knockdown (Fig. 15d to f), indicating that the metastatic change was truly due to CCL9 expression levels rather than an off-target effect.

These results further demonstrated that RAW264.7 cells were a good substitute for primary Gr-1+CD11b+ cells since we could achieve a more efficient and varied knockdown in RAW264.7 cells (Fig. 16d). More importantly, RAW264.7 cells and Gr-1+CD11b+ cells resulted in a very similar level of CCL9-mediated tumor promotion (Fig. 15c, 16 b, e, and f). Together, our data suggested a unique function of myeloid cell-derived CCL9 to promote cancer metastasis.

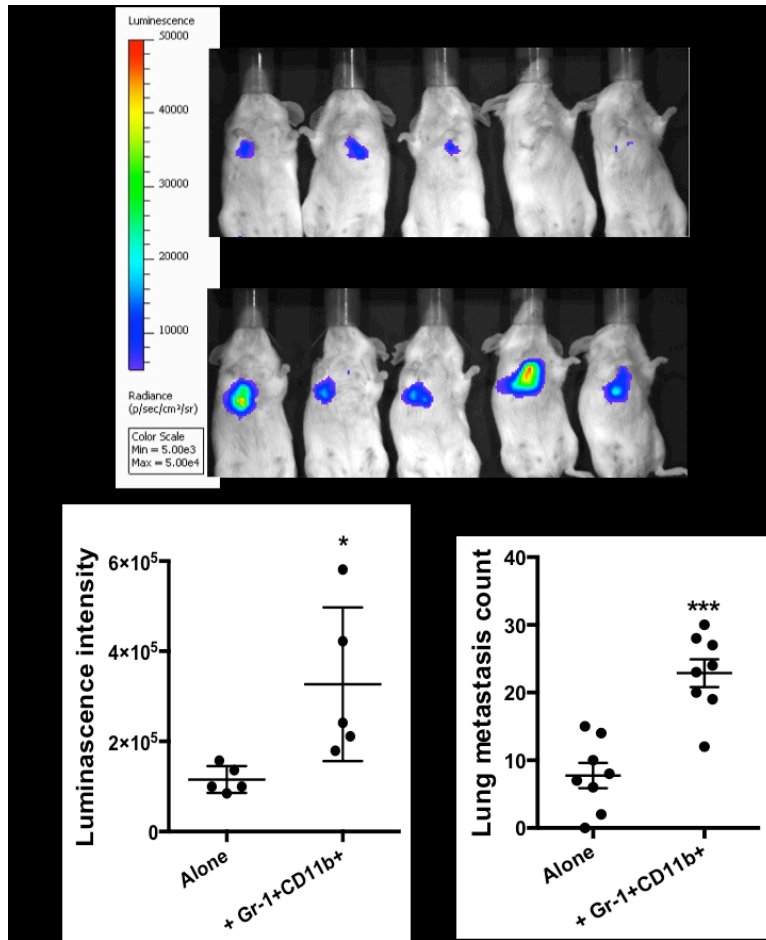


Figure 14. Gr-1+CD11b+ cells promoted tumor metastasis.

(a) Luciferase live imaging 7 days after tail vein co-injection of 5×10^5 4T1-luc with 1×10^6 Gr-1+CD11b+ cells. Mice n=5 for each group. (b) Quantitative data of (a). (c) Number of lung metastasis resulting from co-injection of 5×10^5 67NR tumor cells with 1×10^6 Gr-1+CD11b+ cells through the tail vein. Lungs were harvested 21 days later. *P<0.05, ***P<0.001

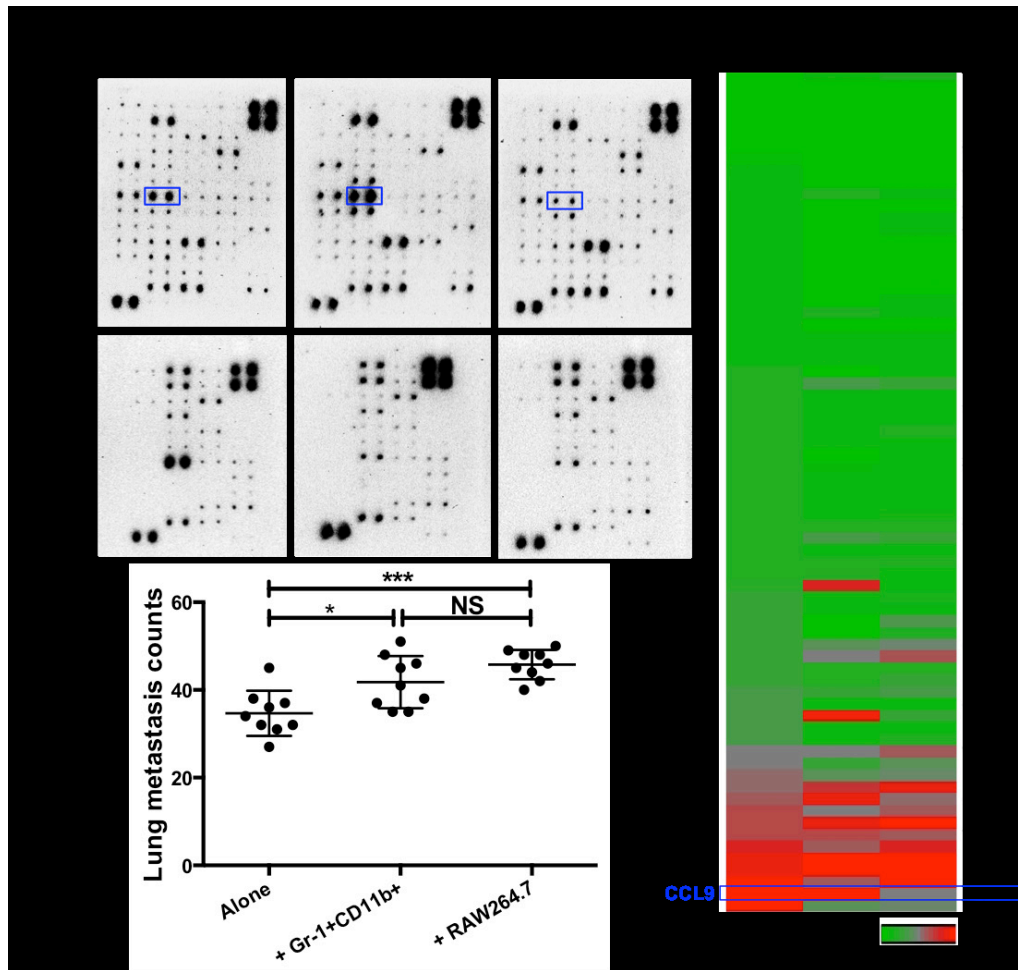


Figure 15. Similar cytokine profile of splenic Gr-1+CD11b+ cells, RAW264.7 cells, and 32DC13 cells.

(a) Cytokine protein array of primary Gr-1+CD11b+ cells (sorted from spleens of tumor-bearing mice), RAW264.7 macrophage cell line, and 32Dcl3 neutrophil cell line, after co-culture with 4T1 tumor-conditioned media overnight. Blue boxes on blots indicate CCL9 expression. **(b)** Heat map based on semi-quantitative data from (a). Dot density was normalized to control dots. Blue box indicated CCL9 expression. **(c)** Lung metastasis of 4T1 cells co-injected with Gr-1+CD11b+ or RAW246.7 cells. n=9-10 mice per group. *P<0.05, ***P<0.001

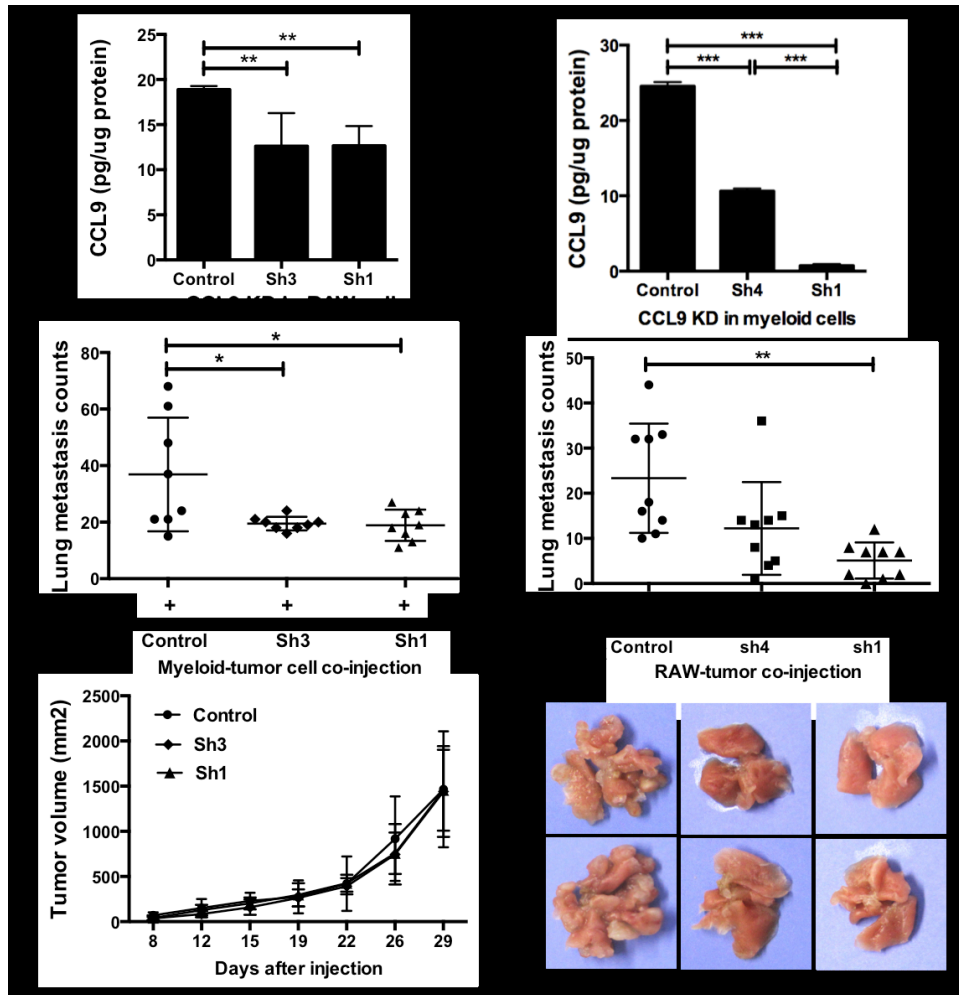


Figure 16. Decreased lung metastasis upon CCL9 knockdown in RAW264.7 or Gr-1+CD11b+ cells.

(a) CCL9 expression after knockdown in RAW264.7 cells. (b) Lung metastasis of 67NR tumor cells co-injected with RAW264.7 cells deficient in CCL9 expression. (c) Primary tumor size when 67NR cells were co-injected with CCL9-deficient Gr-1+CD11b+ cells into mammary fat pad. (d) CCL9 expression after knockdown in primary Gr-1+CD11b+ cells. (e) Lung metastasis of 67NR tumor cells co-injected with CCL9-deficient Gr-1+CD11b+ cells. (f) Representative pictures of (e). For both (a) and (d), CCL9 expression was detected by ELISA. Mice received tail vein (b)(e)(f) or mammary fat pad (c) co-injection of 5×10^5 67NR and 2×10^5 RAW264.7 cells or 1.5×10^6 Gr-1+CD11b+ cells. Mice were euthanized 4 weeks after cell injection. Mice n=8-9 for each group. *P<0.05, **P<0.01, ***P<0.001.

CCR1 mediates CCL9 signaling and promotes tumor metastasis

CCR1 was reported as the sole receptor mediating CCL9 signaling (Kitamura et al., 2010; Kitamura et al., 2007; Lean et al., 2002; Zhao et al., 2003). As described in Chapter III, we found that CCR1 was expressed by tumor cells and the expression level correlated with cancer metastasis (Fig. 10). To examine whether CCR1 was important for lung metastasis, we sorted CCR1⁺ tumor cells and tried to expand them *in vitro*. However, the majority of CCR1⁺ cells lost CCR1 expression during culture. We then overexpressed CCR1 in low metastatic 67NR and B16F1 cells (Fig. 17a and c). Mice bearing 67NR-CCR1 or B16F1-CCR1 tumor cells showed increased lung metastasis compared to 67NR-control or B16F1-control cells (Fig. 17b and d). Highly metastatic cell lines 4T1 or B16F10 served as positive controls (Fig. 17)

To further investigate whether CCL9 signaled through CCR1 to promote cancer metastasis, we co-injected CCL9-deficient RAW264.7 cells with 67NR-CCR1 cells. Consistently, CCL9 knockdown in RAW264.7 cells decreased tumor metastasis and CCR1 overexpression in tumor cells increased tumor metastasis when co-injected with RAW264.7 cells retained a high level of CCL9 expression (Fig. 18). Importantly, CCR1 overexpression in 67NR cells did not rescue the metastasis deficiency in the context of myeloid-specific CCL9 knockdown. This result indicated that CCL9, but not other CC cytokine family members, was likely the major cytokine signaling through the CCR1 receptor to promote metastasis. This finding was consistent with our earlier observation that CCL9 was the most up-regulated cytokine in myeloid-tumor co-culture (Fig. 5).

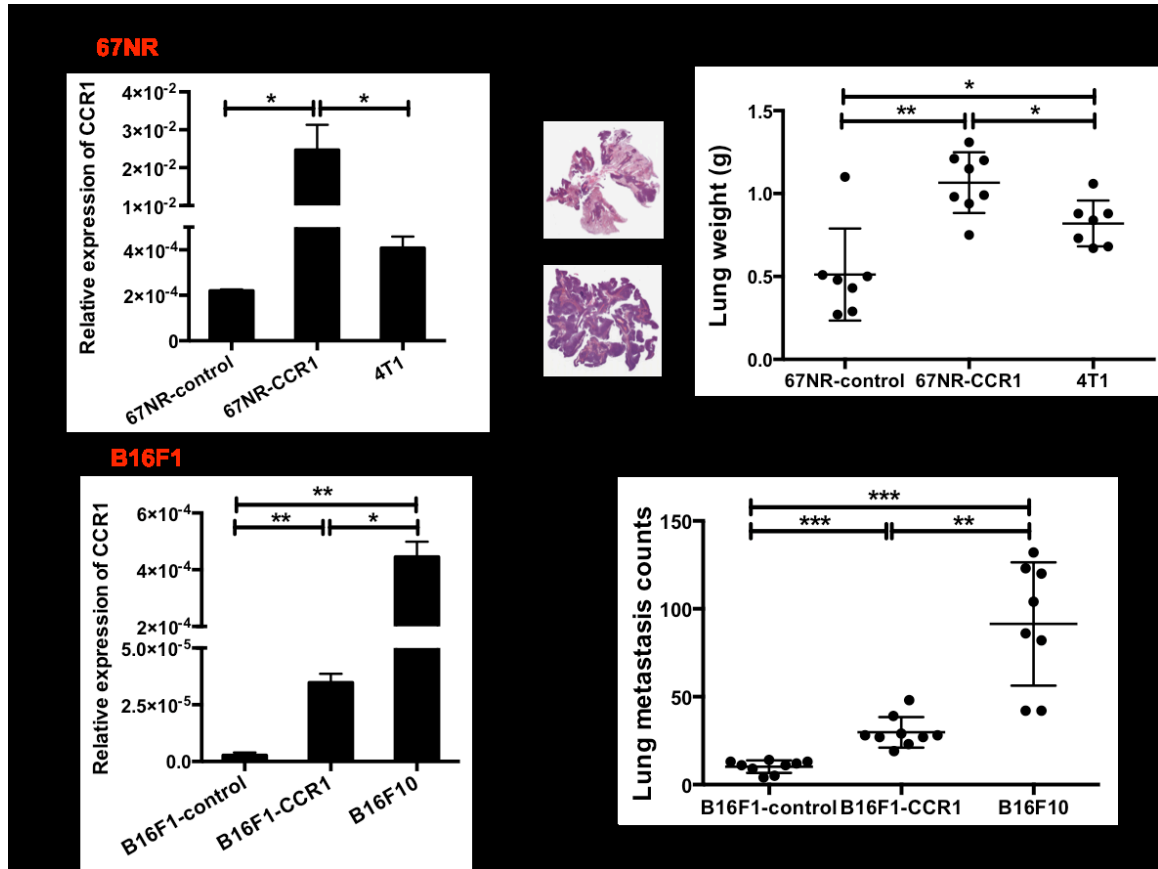


Figure 17. Overexpression of CCR1 increased lung metastasis.

(a) qRT-PCR showing CCR1 overexpression in 67NR cells. (b) Lung metastasis of 67NR cells with CCR1 overexpression (67NR-CCR1) and control (67NR-control). Mice received tail vein injection of 67NR-CCR1, 67NR-control, or 4T1 cells; the lungs were harvested 21 days later. Left panel: representative H&E sections of mouse lungs; Right panel: lung weights, n=7-8 mice for each group. (c) qRT-PCR showed CCR1 overexpression in B16F1 cells. (d) Number of lung metastasis in mice that received tail vein injection of B16F1-CCR1, B16F1-vec, or B16F10 cells. Lungs were collected 21 days after tail vein injection and subjected to whole lung mounting procedure. n=8-9 mice for each group. *P<0.05, **P<0.01, ***P<0.001.

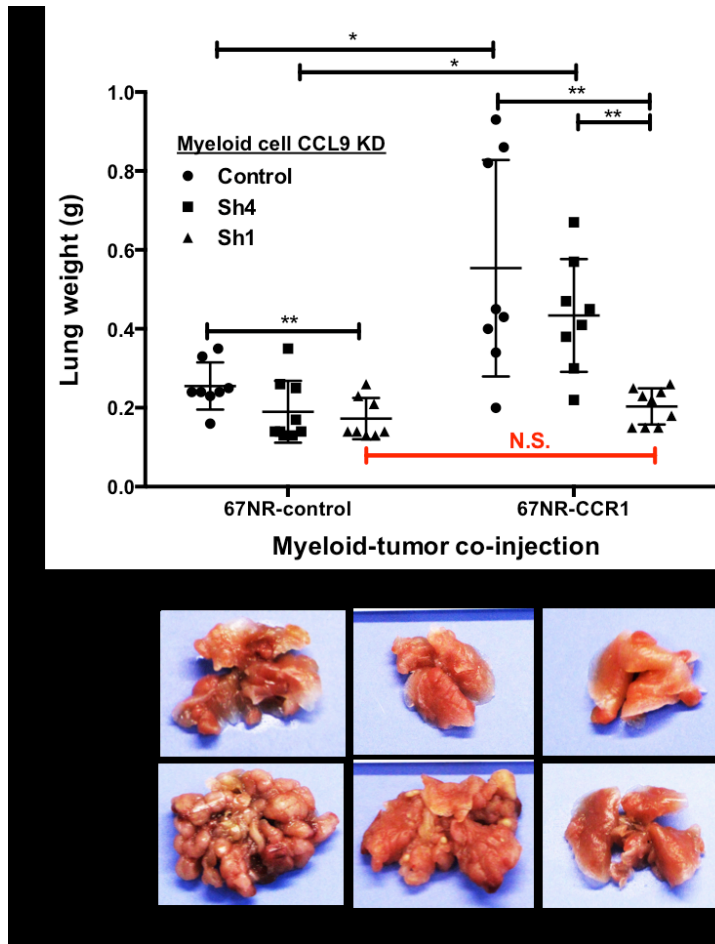


Figure 18. CCL9/CCR1 promoted tumor metastasis.

(a) Lung weights and **(b)** representative pictures of tumor-bearing mice that received the co-injection of 67NR-CCR1 cells or its control with RAW264.7 cells with CCL9 knockdown. Mice were euthanized 4 weeks after injection. $n=9$ mice for each group.

* $P < 0.05$, ** $P < 0.01$.

CCL9 promotes tumor cell survival

When 4T1 cells were introduced into the venous circulation of mice, the majority of 4T1 tumor cells died within 24 hours after arrest at the secondary site (Fig. 19). To investigate the effect of myeloid-derived CCL9 on tumor cell survival in the premetastatic lung, we utilized fluorescence imaging of single cell video microscopy (SCVM) and pulmonary metastasis assay (PUMA). SCVM detected single tumor cell survival *in vivo*, which allowed us to examine the effect of myeloid CCL9 on tumor cell survival. Lungs subjected to SCVM were further analyzed using the TUNEL assay to detect apoptotic tumor cells. PUMA evaluated tumor cell colonization *ex vivo* in the cultured lung slice 7-14 days after tail vein co-injection of myeloid and tumor cells (Mendoza et al., 2010).

We used 67NR-GFP or B16F1-GFP cells in these assays, as they are more vulnerable to the effects of CCL9 knockdown in myeloid cells compared to highly metastatic 4T1-GFP or B16F10-GFP cells. Using SCVM and TUNEL assays, we found that CCL9 knockdown in RAW264.7 cells decreased the survival and increased apoptosis of 67NR-GFP cells (Fig. 20a) and B16F1-GFP tumor cells (Fig. 20c). CCL9 knockdown in RAW264.7 cells also decreased metastatic colony formation of 67NR-CCR1 (Fig. 21a) and B16F1 cells (Fig. 21c), as demonstrated by PUMA. These results were further validated through CCL9 knockdown in primary Gr-1⁺CD11b⁺ myeloid cell, which increased tumor cell apoptosis (Fig. 20b) and decreased tumor cell survival and metastatic colony formation (Fig. 20b and 21b).

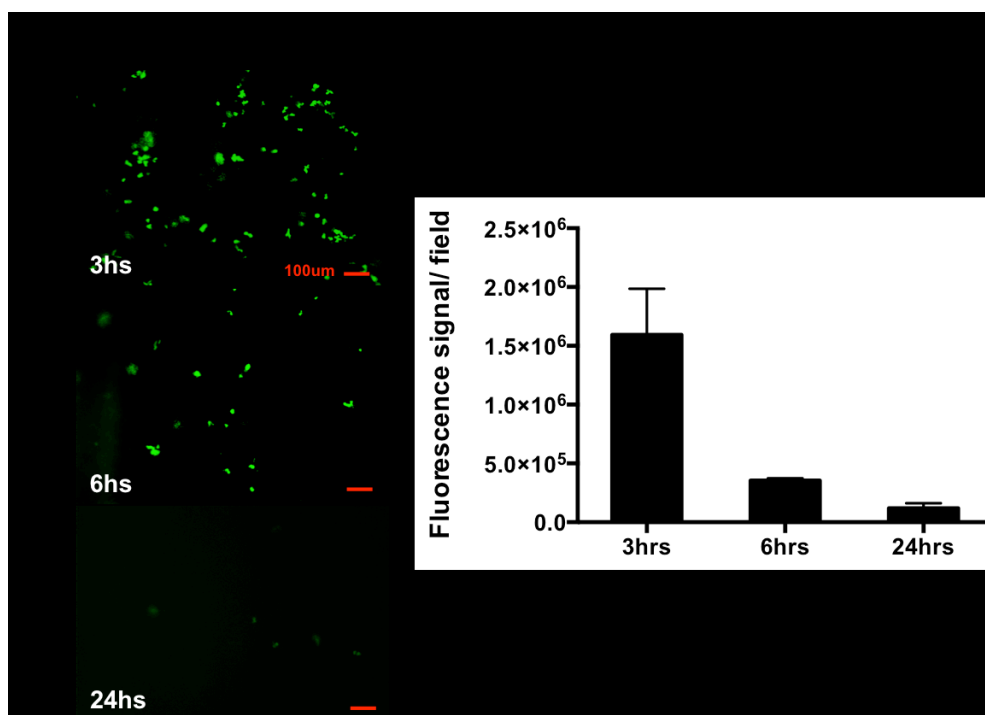


Figure 19. The majority of tumor cells died within 24 hours after tumor inoculation. 5×10^5 4T1-GFP cells were tail vein injected into BALB/c mice. Lungs were taken out after indicated hours for confocal imaging. **(a)** Representative confocal images at each indicated time point. **(b)** Quantitative data showed as fluorescence signal per field. Each time point result was the average of 6 random fields under 20× magnification. 3 mice per time point were used.

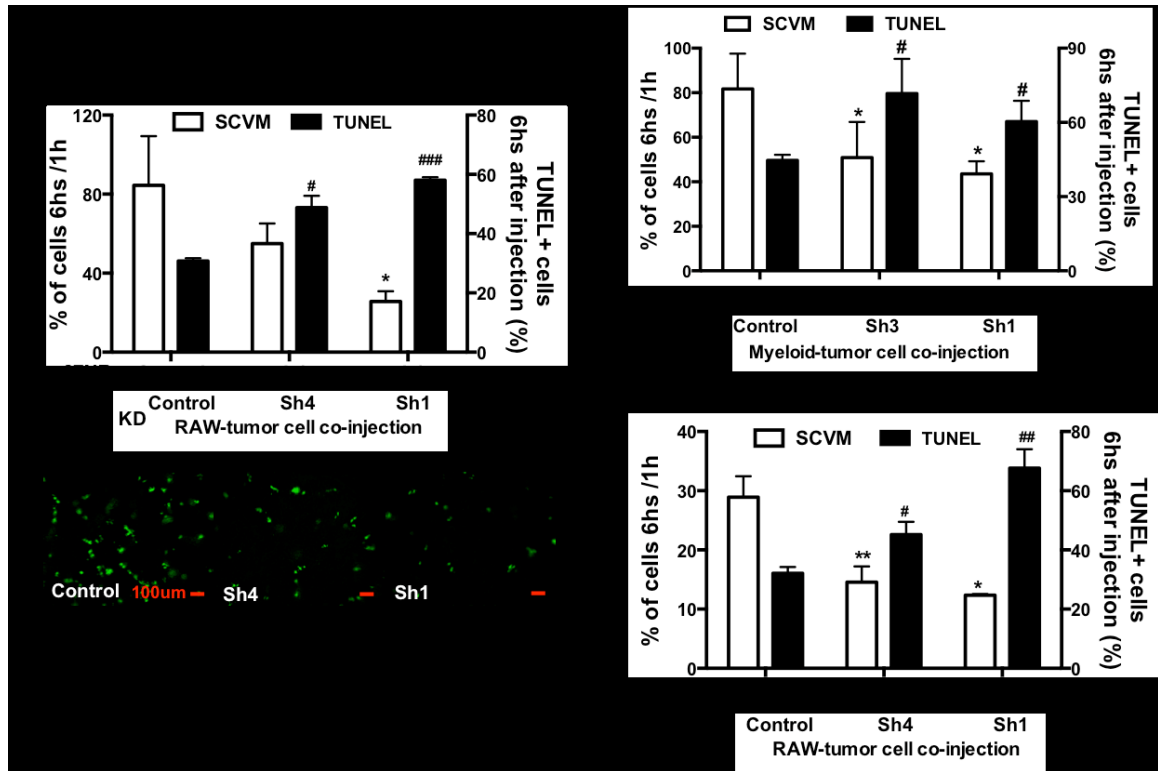


Figure 20. CCL9 knockdown in myeloid cells decreased tumor cell survival and increased tumor cell apoptosis *in vivo*.

SCVM for tumor cell survival (left y-axis) and TUNEL assay for tumor cell apoptosis (right y-axis). **(a)** GFP labeled 67NR cells were co-injected with 2×10^5 RAW264.7 cells with or without CCL9 knockdown. Upper panel: quantitative data. Lower panel: representative SCVM pictures 6 hours after injection. **(b)** GFP labeled 67NR cells were co-injected with 1.5×10^6 Gr-1+CD11b+ cells with or without CCL9 knockdown. **(c)** GFP labeled B16F1 cells were co-injected with 2×10^5 RAW264.7 cells with or without CCL9 knockdown. Ten fields for each mouse lung were examined. Fluorescent signals at 6 hours were normalized to 1 hour (baseline) signals. $n=3-4$ mice per group. Fixed lung tissues were then stained with GFP to label the tumor cells and TUNEL assay to identify apoptotic cells. * or # $P < 0.05$, ** or ## $P < 0.01$, ### $P < 0.001$.

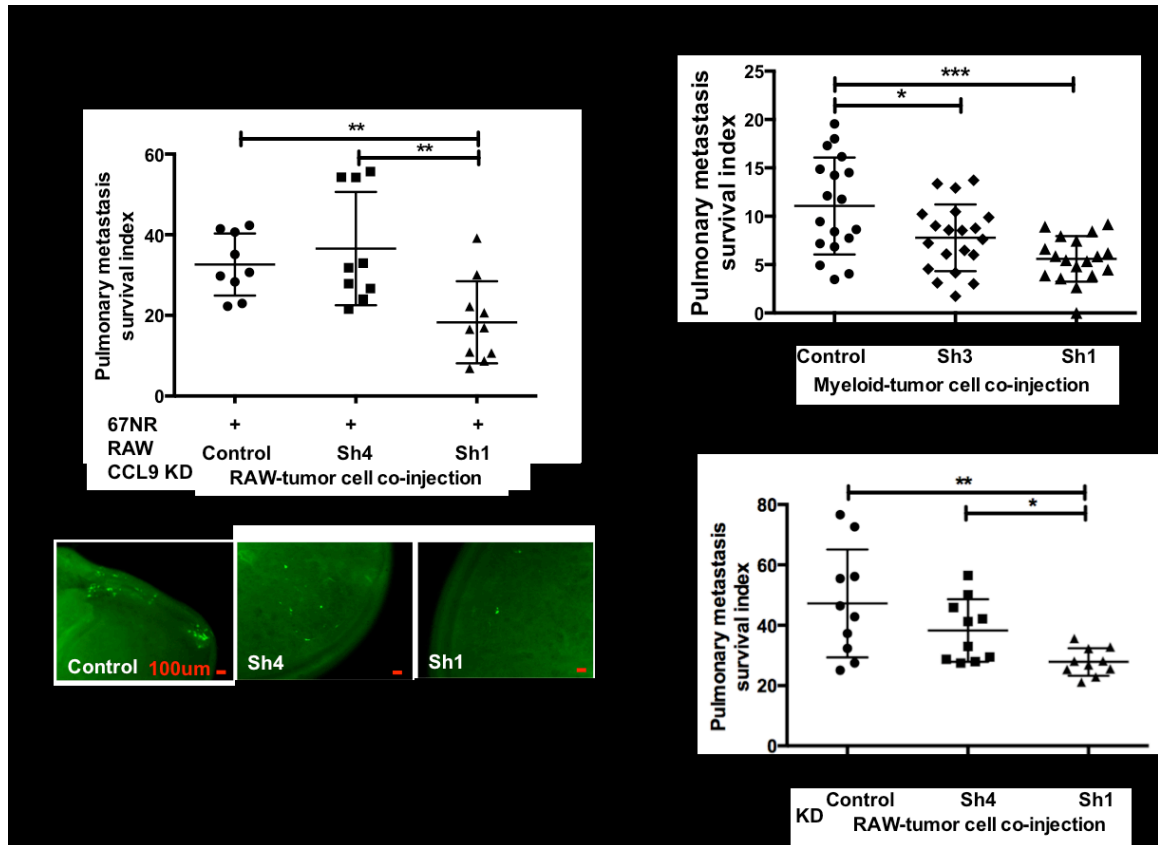


Figure 21. CCL9 knockdown in myeloid cells decreased tumor cell colonization. PUMA for tumor cell survival and metastasis. **(a)** GFP labeled 67NR cells were co-injected with 2×10^5 RAW264.7 cells with or without CCL9 knockdown. Upper panel: quantitative data. Lower panel: representative PUMA pictures 7 days after lung section culture. **(b)** GFP labeled 67NR cells were co-injected with 1.5×10^6 Gr-1+CD11b+ cells with or without CCL9 knockdown. **(c)** GFP labeled B16F1 cells were co-injected with 2×10^5 RAW264.7 cells with or without CCL9 knockdown. Signals obtained on day 7 were normalized to those from day 0 (baseline) and presented as pulmonary metastasis index. Three mice each group, 3-4 lung pieces each mouse. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

CCL9 does not affect tumor cell extravasation

Tumor cell extravasation had been thought to be an important step prior to tumor cell survival in the distant metastatic organs. Tumor cells unable to extravasate died in blood vessels, while those that successfully extravasated were more likely to survive (Valiente et al., 2014). Thus, we aimed to understand whether increased tumor cell survival was due to a direct CCL9 effect or through an increased extravasation. To answer this question, we injected 67NR-GFP cells together with fluorescent dextran through the tail vein. We took confocal images of lungs 6 hours after injection and analyzed the data by counting green fluorescent 67NR-GFP tumor cells against a red vascular map (Dextran labeled) (Fig. 22b). We found that Gr-1+CD11b+ cells promoted tumor cell extravasation, which was consistent with a previous publication (Yang et al., 2008a). However, knockdown of CCL9 in Gr-1+CD11b+ cells did not affect tumor cell extravasation compared to shLuc control group (Fig. 22).

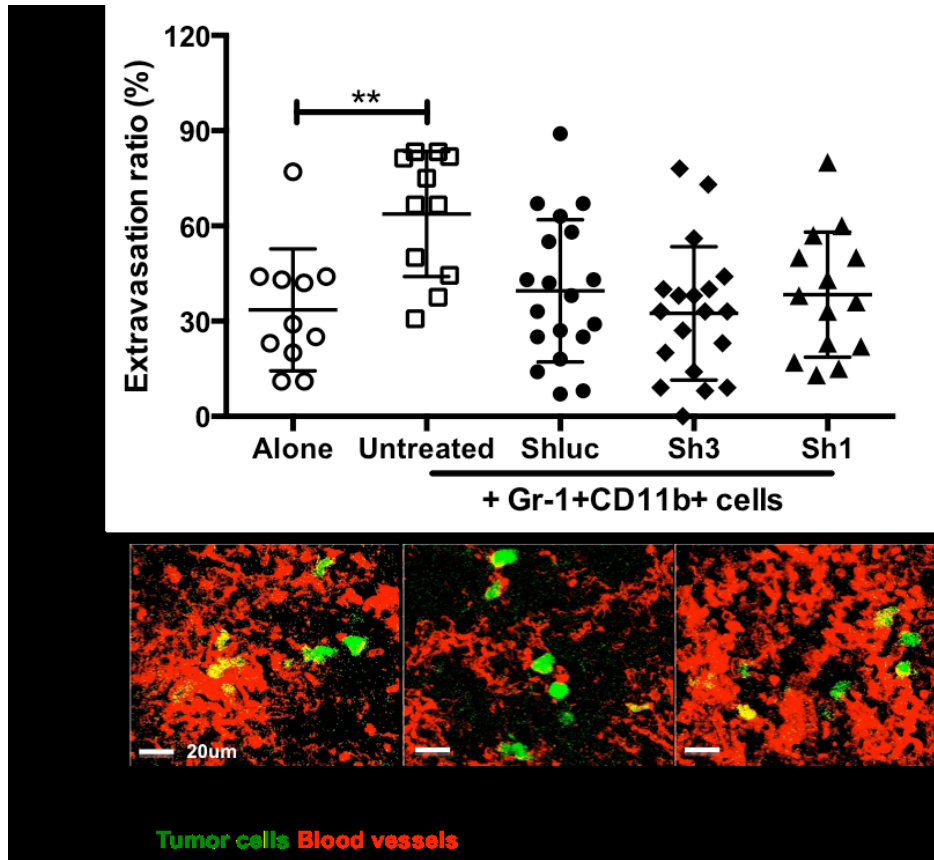


Figure 22. CCL9 did not affect tumor cell extravasation.

Confocal images were taken with indicated cell injection through the tail vein. Blood vessels were labeled with dextran. **(a)** Percentage of extravasated tumor cells over total cells observed. **(b)** Representative pictures of (a). **P<0.01.

Molecular mechanisms by which CCL9 promotes tumor cell survival

To determine the effect of CCL9 on tumor cell survival *in vitro*, we continued to use the low metastatic 67NR mammary tumor cells and B16F1 melanoma cells that were more sensitive to apoptosis induction upon serum starvation and glucose free culture conditions. Recombinant mouse CCL9 (rmCCL9) decreased the number of 7AAD+/AnnexinV+ 67NR and B16F1 cells, while CCL9 neutralization had the opposite effect (Fig. 23a and b). rmCCL9 also increased the survival of 67NR cells when cultured in glucose free conditions (Fig. 23c). Furthermore, 4T1 cells treated with doxorubicin showed an increased survival upon rmCCL9 treatment (Fig. 23d and e). 67NR cells were not used as they were mostly killed by doxorubicin treatment.

We next addressed the molecular mechanisms underlying CCL9 promotion of tumor cell survival. We firstly looked into AKT pathway, as this was reported to promote lung cancer cell survival upon CCL5/CCR1 activation (Huang et al., 2009). We found that rmCCL9 increased phosphorylated-AKT (p-AKT), whereas CCL9 neutralizing antibody (NeuAB) decreased p-AKT expression (Fig. 24a). Secondly, we looked into the caspase-dependent apoptosis pathway (Fig. 24c). The apoptosis was likely independent of the caspase cascade as there was no change in cleaved caspase 3 when treated with rmCCL9 or CCL9 NeuAB (Fig. 24a). We then looked into an important non-canonical, poly (ADP-ribose) polymerase (PARP)-dependent apoptosis pathway (Schreiber et al., 2006) (Fig. 24d). rmCCL9 decreased PARP expression in 67NR cells whereas CCL9 NeuAB elevated its expression in 67NR cells that were cultured in myeloid-tumor

co-culture supernatant (Fig. 24a). Immunofluorescence (IF) staining of PAR, the substrate of PARP, showed that rmCCL9 decreased PAR, whereas CCL9 NeuAB elevated PAR in B16F1 cells cultured in myeloid-tumor co-culture supernatant, supporting a role of the PARP in CCL9 promotion of tumor cell survival (Fig. 24b). BCL-2, a molecular inhibitor of PARP pathway, was also elevated when rmCCL9 was applied and decreased when CCL9 NeuAB was added (Fig. 24a). Together, these data indicated that CCL9 activates the AKT cell survival pathway and inhibits the PARP-dependent apoptosis pathway (Fig. 24e).

The autocrine effects of CCL9/CCR1 in Gr-1+CD11b+ cells

CCL9 had been reported to inhibit apoptosis in osteoclasts, one of the myeloid cell types (Okamatsu, 2004). We therefore asked whether CCL9 had an effect on Gr-1+CD11b+ cell survival. As expected, CCL9 neutralization increased apoptosis of Gr-1+CD11b+ cells cultured in tumor-conditioned media (Fig. 25a). In addition, knockdown of CCR1 in RAW264.7 cells decreased their CCL9 production (Fig. 25b), suggesting an autocrine effect mediated by the CCL9/CCR1 axis.

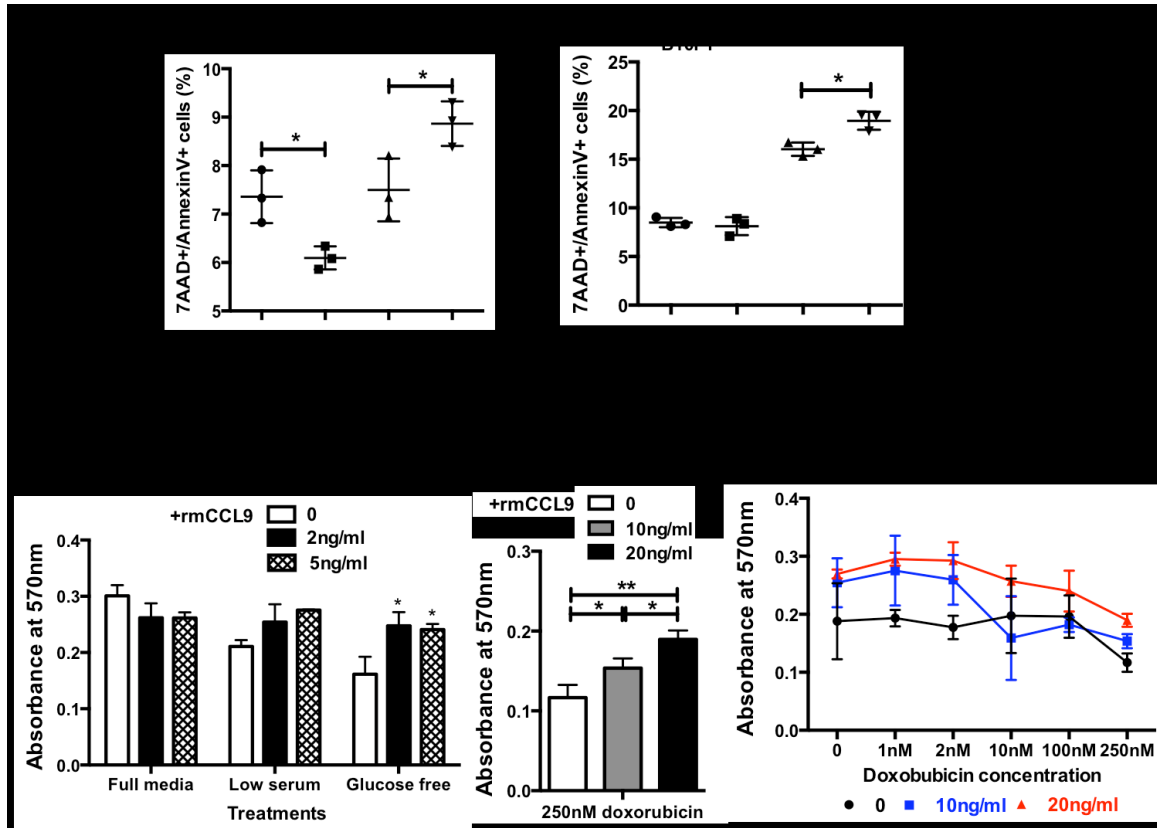


Figure 23. CCL9 promoted tumor cell survival *in vitro*.

Flow cytometry analysis of (a) 67NR and (b) B16F1 cells stained with Annexin V and 7AAD after co-cultured in the indicated conditions. MTT assay showing tumor cell survival when treated with rmCCL9. (c) 67NR cells under low serum and glucose free culture conditions, as well as (d) (e) 4T1 cells with anti-cancer drug doxorubicine. rmCCL9 was added to 4T1 cells before overnight treatment of doxorubicine. The experiment were repeated 2-3 times, data shown was a representative performed with triplicates for each group. * $P < 0.05$, ** $P < 0.01$.

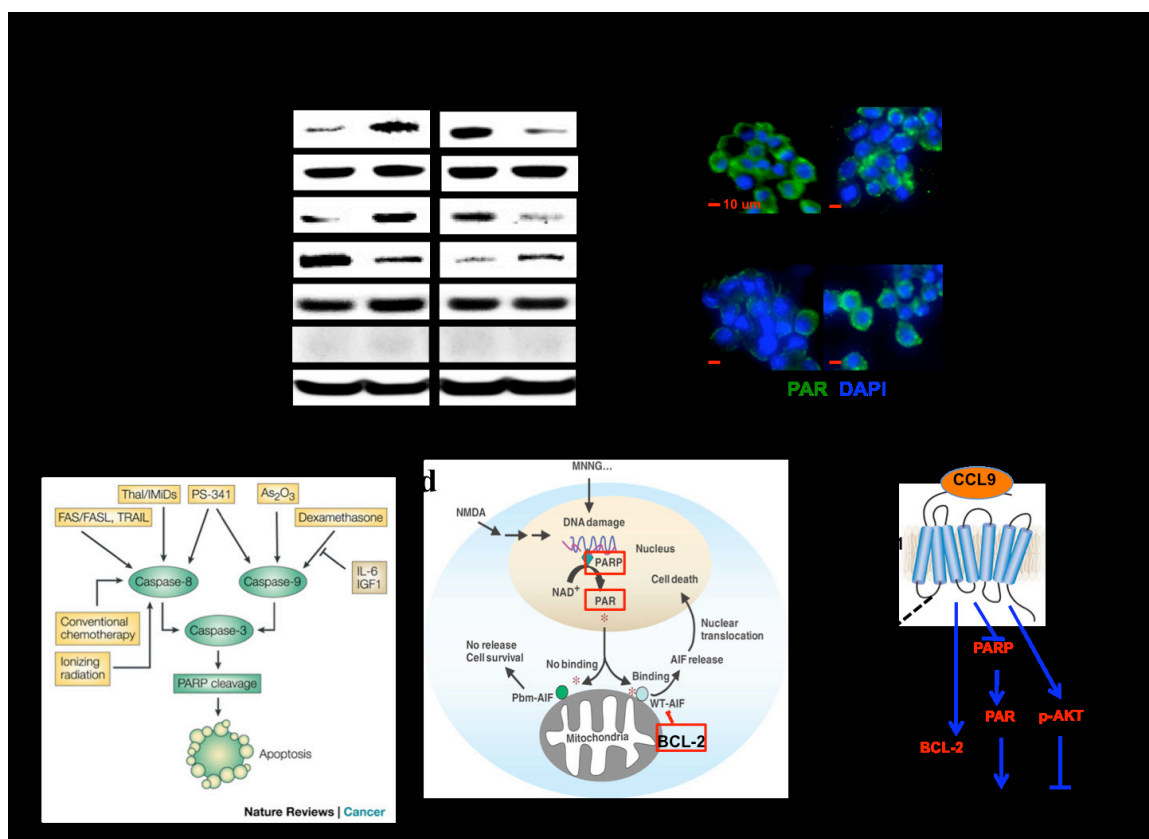


Figure 24. Molecular mechanisms of CCL9-mediated tumor cell survival.

(a) Western blots of P-AKT, AKT, BCL-2, PARP, caspase 3, cleaved caspase 3, as well as β -actin. 67NR cells were firstly starved under low serum (1%) for 24 hours and then treated as indicated. **(b)** PAR and nuclei fluorescence staining (PAR in green, with nuclei in blue) of serum starved B16F1 cells treated with rmCCL9 or CCL9 NeuAB when cultured in myeloid-tumor co-culture supernatant. **(c)** Caspase dependent apoptosis pathway. Figure adapted from: Nat Rev Cancer. 2002 Dec;2(12):927-37. **(d)** PARP-dependent apoptosis pathway. Figure adapted from: Sci Signal. 2011 Apr 5;4(167):ra20. doi: 10.1126/scisignal.2000902. Molecules labeled with red boxes are the ones altered when treated with rmCCL9 or CCL9 NeuAB. **(e)** Schematic pathways for tumor cell survival and apoptosis regulated by CCL9. Abbreviation: rmCCL9: recombinant mouse CCL9; CoSN: myeloid-tumor co-culture supernatant; CCL9 NeuAB: CCL9 neutralizing antibody.

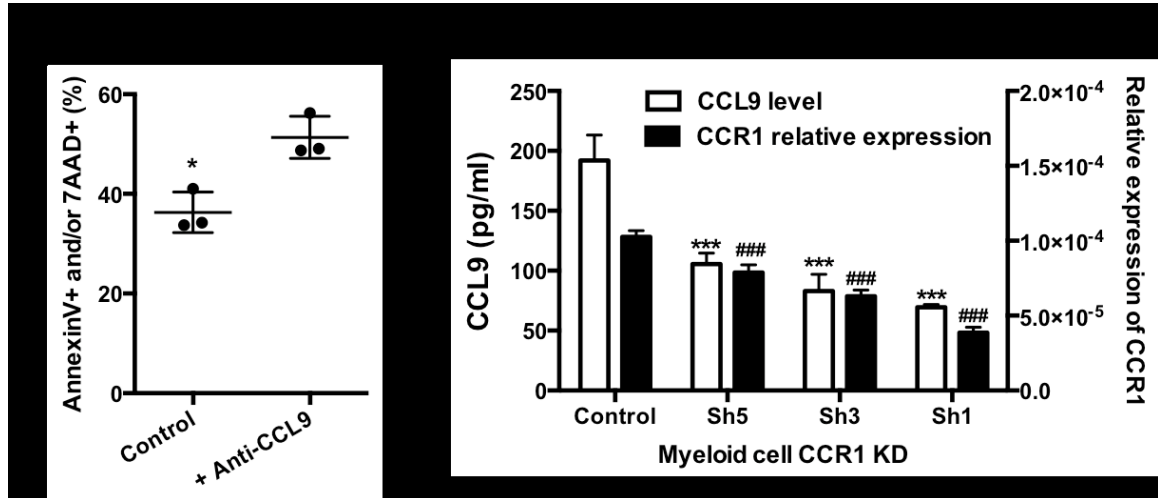


Figure 25. Autocrine effect of CCL9/CCR1 on Gr-1+CD11b+ cells.

(a) Flow cytometry analysis of AnnexinV and 7AAD of stained Gr-1+CD11b+ cells cultured in myeloid-tumor co-culture supernatant and treated with CCL9 neutralizing antibody. Three mice were used for each group. **(b)** CCL9 expression (ELISA, left Y-axis) and relative expression of CCR1 (RT-qPCR, right Y-axis) upon CCR1 knockdown in RAW264.7 cells. * P<0.05, ** P<0.01, *** or ### P<0.001

Discussion

RAW264.7 cells as a substitute for Gr-1+CD11b+ cells

RAW264.7 cells showed a very similar cytokine profile when compared with Gr-1+CD11b+ cells, especially in the CCL9 expression levels (Fig. 15). With only a small amount of variations in other cytokine expressions, RAW264.7 cells provided an effective model for us to investigate CCL9 function using the CCL9 knockdown approach. In addition, RAW264.7 cells had a very similar tumor-promoting function as Gr-1+CD11b+ cells (Fig. 15) and exhibited decreased metastasis with CCL9 knockdown (Fig. 16). In the efforts to understand how CCL9 promotes metastasis, we performed SCVM, TUNEL, and PUMA to test tumor cell survival and colonization in both RAW264.7 and Gr-1+CD11b+ cells with similar results.

The biggest advantage of using RAW264.7 over Gr-1+CD11b+ cells was that we achieved a much higher degree of knockdown in RAW264.7 cells. RAW264.7 cells of CCL9 knockdown had a puromycin resistant gene, which was co-expressed with shRNAs. As a result, the CCL9 expression level was stable in these cells as they were under drug selection in culture. The different degrees of CCL9 expression helped us to understand whether decreased metastasis was due to CCL9 expression level or an off-target effect.

There was a possibility that our results obtained from RAW264.7 cells are different from Gr-1+CD11b+ cells. To address this in future, using conditional CCL9 KO mice would be the most ideal choice, but myeloid specific CCL9 knockout mice were not readily available. Although the total KO mice were available from the Knockout Mouse

Project (KOMP), it required cryorecovery from frozen embryos, which would take at least nine months to complete. In addition, not only the cost was rather high, but also we would need to cross breed for at least 5 generations to match our study models in BALB/c and C57BL/6 backgrounds. Therefore, considering the cost and time constraints, we were unable to utilize these mouse models in my studies.

Using lower metastatic 67NR and B16F1 cells to study the tumor-promoting function of CCL9 and CCR1

We chose to use low metastatic 67NR and B16F1 cells when we tested whether CCL9 could increase metastasis and survival, because the basal level of metastasis in 4T1 and B16F10 cells was already high (Fig. 17). These experiments included all the co-injections (Fig. 16, 20 to 22) and co-cultures (Fig. 23 and 24) of tumor and Gr-1+CD11b+ cells with or without CCL9 knockdown. In addition, in the experiments utilizing overexpression of CCR1 in tumor cells, we used 67NR/B16F1 since 4T1/B16F10 cells had intrinsically high level of CCR1 (Fig. 17).

CCL9 neutralization or knockdown in primary myeloid cells as an option for metastasis treatment

We had explored extensively the possibility of treating mice with CCL9 NueAB. Due to the prohibitive cost, we were unable to immediately start this experiment, and so will be one of the future directions to explore.

However, modification of protein expression in primary cells was achievable. CCL9 knockdown from Gr-1+CD11b+ cells clearly decreased tumor metastasis (Fig. 16). It would be very interesting to further explore the clinical applications of this result. Potential experiments could include introducing CCL9-deficient Gr-1+CD11b+ cells to lethally irradiated tumor-bearing mice. We have already successfully knocked down CCL9 in Gr-1+CD11b+ cells by electroporation. Our lab also has extensive experience in bone marrow transplantation of irradiated mice, in which majority of white blood cells are dysfunctional. We can use this approach to test whether CCL9-deficient Gr-1+CD11b+ cells block the metastasis promoting effect as a promising pre-clinical study. We could also treat tumor-bearing mice with CCL9 shRNA driven by the CD11b promoter *in vivo*. Our lab have developed the system where TGF- β shRNA that under the myeloid cell promoter CD11b were tail vein injected into tumor-bearing mice for three weeks. TGF- β shRNA reprogrammed these cells from type 2 into type 1 that displays tumor-inhibiting function to reduce metastasis. CCL9 that identified in this study is a new promising target and specific knockdown in myeloid cells *in vivo* may inhibit metastasis as an indication for clinical treatment.

CCL9/CCR1 promotes metastasis by enhancing tumor cell survival

To our best knowledge, we first time identified CCL9 as a key player in promoting cell survival in the premetastatic lung, for both tumor and Gr-1+CD11b cells (Fig. 20, 23, and 25). In a previous publication, the authors showed that iMCs formed a permissive

premetastatic niche for the incoming tumor cells (Kaplan et al., 2005). However, a co-localization of tumor and Gr-1+CD11b+ cells in the premetastatic lung was not observed in our tumor models. CCL9 was significantly induced in the premetastatic lung microenvironment and enhanced tumor cell survival without a spatial restriction. In other words, overlapping of tumor and Gr-1+CD11b+ cells was unnecessary for CCL9 to function as a metastasis promoter.

Our results add a more complete understanding of CCL9/CCR1 function. A previous publication demonstrates CCL9/CCR1 as cell attractant molecules. CCL9 recruits CCR1+ iMCs, which secrete MMP2 and MMP9 to promote tumor cell invasion at the tumor invasive front (Kitamura et al., 2007). Their study and ours indicate that CCL9/CCR1 is critical for both tumor cell invasion and survival. Thus, CCL9/CCR1 may serve as a more promising therapeutic target.

Gr-1+CD11b+ cells are metastasis-promoting

Our study showed a tumor-promoting function of Gr-1+CD11b+ cells. This was consistent with the metastasis-promoting effect of Gr-1+ inflammatory monocytes (Qian et al., 2011), immature myeloid cells (Hiratsuka et al., 2006), and VEGFR1+ myeloid progenitor cells (Kaplan et al., 2005).

Our result was different from a recent publication, which indicated that Ly6G+CD11b+ cells exhibit an anti-tumor function (Granot et al., 2011). These differences most likely reflected the context-dependent functionality of myeloid cells,

such as time and spatial factors in mouse models. In their study, the authors showed that neutralization of Ly6G⁺CD11b⁺ cells promoted metastasis. Such neutralization was only successful before, but not after, the premetastatic stage (day 14 after tumor inoculation). This might be due to the fact that Ly6G⁺ cells only become tumor-promoting around day 14 when primary tumor secreted factors circulate to and function in the secondary organ. In support of this idea, we found that CCL9 only became significantly upregulated at the premetastatic lung stage (Fig. 8)

The autocrine effect of CCL9/CCR1

CCL9 was expressed by Gr-1⁺CD11b⁺ cells, which also expressed high level of CCL9 receptor CCR1 (Fig. 25). In one previous publication, CCL9/CCR1 was reported to recruit myeloid cells into primary tumor tissue (Kitamura et al., 2007). Here we found a novel function of this cytokine/receptor axis to promote the cell survival of Gr-1⁺CD11b⁺ cells. Gr-1⁺CD11b⁺ cells are an important component in supporting incoming metastatic tumor cells, thus the survival of those cells is critical.

CCL9/CCR1 inducing CCL9 secretion is an important mechanism to further amplify CCL9 signal to promote the survival of both cell types. Neutralization or knockdown of CCL9 may have a compounded effect on both tumor and Gr-1⁺CD11b⁺ cells, killing two birds with one stone.

CHAPTER V

CCL9 IS A CRITICAL EFFECTOR OF MYELOID TGF- β SIGNALING THAT PROMOTES METASTASIS

Introduction

TGF- β displays activity as both a metastasis promoter and a suppressor. Large amounts of evidence suggest that TGF- β signaling functions as a metastasis suppressor in epithelial cells (Forrester et al., 2005; Kitamura et al., 2007), fibroblasts (Hembruff et al., 2010), and T cells (Kim et al., 2006), as the deletion of TGF- β pathway molecules in these cell types promotes tumor progression. Distinct from the cell types discussed above, myeloid specific TGF- β functions to promote metastasis. Our lab has previously discovered a significantly decreased metastasis when TGF- β receptor II (T β RII) is deleted specifically in the myeloid cell population (Fig. 26a). This is obtained by using mice with a targeted deletion of Tgfbr2 in myeloid cells (Tgfbr2^{MyeKO}). These knockout mice are generated by cross breeding floxed Tgfbr2 (Tgfbr2^{flox/flox}) with LysM-Cre transgenic mice, as LysM is a myeloid specific promoter (Pang et al., 2013).

Cytokines are the key effectors of TGF- β signaling pathway. TGF- β promotes the expression of chemokines that attract inflammatory cells into the tumor microenvironment (Bierie et al., 2009; Ijichi et al., 2011; Pickup et al., 2013; Yang et al., 2008a). For example, deletion of TGF- β in mammary gland epithelial cells increases CXCL5 and CXCL12, also known as stromal derived factor 1, (SDF-1). These cytokines attract CXCR2⁺ and CXCR4⁺ Gr-1⁺CD11b⁺ cells to promote tumor invasion (Yang et al., 2008a). Our own study further supports this idea, a decrease in type 2 cytokines and

increase in type 1 cytokine IFN- γ was found in TGF- β deficient Gr-1+CD11b+ cells (Pang et al., 2013).

Interestingly, we have observed decreased CCL9 expression in TGF- β deficient Gr-1+CD11b+ cells along with significantly decreased tumor metastasis in Tgfb β 2^{MyeKO} mice (Fig. 26 a and b). As shown in Chapter II, CCL9 promotes tumor cell survival and metastasis. This leads us to hypothesize that CCL9 may be an important regulator in myeloid TGF- β pathway to promote metastasis through supporting cell survival. In this chapter, we will test this possibility by overexpressing CCL9 in TGF- β deficient Gr-1+CD11b+ cells, then analyzing metastatic changes.

TGF- β signaling pathways include the canonical SMAD-dependent pathway and non-canonical SMAD-independent pathways (Bierie and Moses, 2006). Myeloid TGF- β is tumor-promoting, as opposed to tumor-suppressing in other reported cell types. This unique functionality in myeloid cells may be due to a different downstream TGF- β pathway from other cell types. In this chapter, we will also look into which downstream molecules are activated to promote the expression of CCL9.

Results

Myeloid TGF- β promotes CCL9 expression

In our recent study on the function of myeloid TGF- β signaling in metastasis, we found a significantly decreased tumor metastasis in $Tgfb2^{MyeKO}$ mice when compared to $Tgfb2^{Flox}$ mice (Fig. 26a). This phenotype was also observed in various tumor models (Pang et al., 2013). T β RII-deficient Gr-1+CD11b+ cells displayed a significantly decreased CCL9 expression in a cytokine protein array (Fig. 26b). We further confirmed these results with ELISA using multiple T β RII-deficient or control tumor-bearing mice (Fig. 26c). In addition, TGF- β neutralizing antibody 1D11 decreased CCL9 expression *in vitro*, as compared to the 13C4 control antibody (Fig. 26d).

CCL9 is a critical effector of the myeloid TGF- β signaling that promotes metastasis

Unlike wild type Gr-1+CD11b+ cells, T β RII-deficient Gr-1+CD11b+ cells failed to support tumor cell survival *in vivo* when examined by SCVM (Fig. 27a). Instead, these cells restored tumor cell apoptosis to a level similar to that of the tumor cells alone group (Fig. 27b). To investigate the importance of CCL9 in TGF- β regulation of myeloid cell function, we overexpressed CCL9 in T β RII-deficient Gr-1+CD11b+ cells (Fig. 27c). Tumor cells showed increased survival and metastasis when co-injected with T β RII-deficient myeloid cells overexpressing CCL9 compared to vector control expression. Overexpression of CCL9 rescued the deficiency in T β RII-deficient myeloid cells and showed no difference with flox control myeloid cells (Fig. 27d). This result

strongly demonstrated that CCL9 was the key effector of myeloid TGF- β signaling to promote metastasis.

Phosphorylated-p38 is a molecular mediator of the myeloid TGF- β pathway that regulates CCL9 expression

We next examined the downstream mediators of the TGF- β pathway that might regulate CCL9 expression. Upon TGF- β treatment *in vitro*, a decreased level of P-p38 and P-SMAD2 to a lesser extent, was observed in T β RII-deficient myeloid cells compared to that of wild type. However, this difference between T β RII-deficient and wild type myeloid cells was not observed in untreated conditions (Fig. 28a).

To investigate the role of p38 in TGF- β regulation of CCL9 production, we cultured the floxed and T β RII-deficient Gr-1+CD11b+ cells in 4T1 tumor-conditioned media that contains high level of TGF- β . These cells were treated with the p38 inhibitor SB203580, which specifically inhibits p38 kinase activity, as shown by inhibited phosphorylation of ATF2, a target of P-p38 (Fig. 28b, right panel). SB203580 reduced CCL9 production specifically in the control but not in T β RII-deficient Gr-1+CD11b+ cells (Fig. 28b, left panel), suggesting P-p38 is critical in regulating TGF- β signaling and CCL9 expression in these immature myeloid cells. Additionally, p38 dominant negative Gr-1+CD11b+ cells showed a decreased CCL9 production compared to those cells from wild type mice (Fig. 28c).

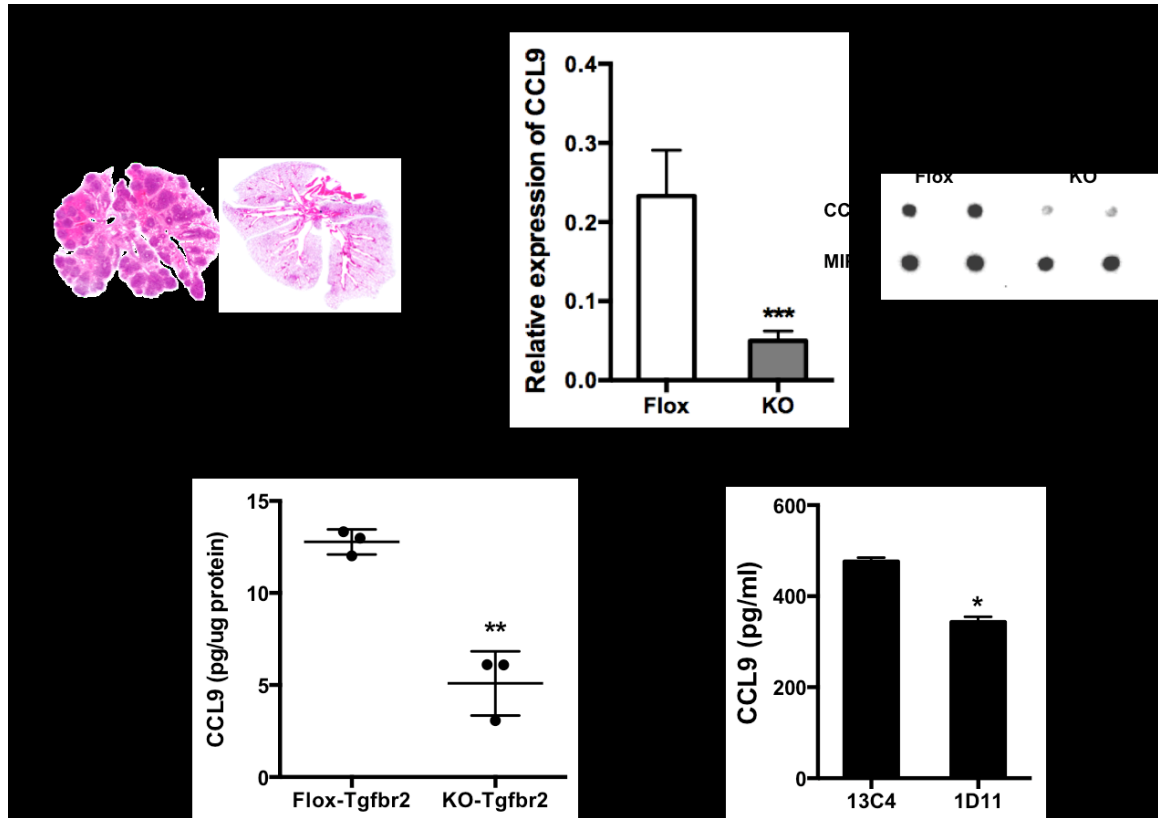


Figure 26. Deletion of myeloid *Tgfb2* decreased lung metastasis and CCL9 expression.

(a) Lung metastasis of *Tgfb2*^{Flox} and *Tgfb2*^{MyeKO} mice (Pang et al., 2013). **(b)** Cytokine protein array indicating CCL9 expression in floxed or TβRII-deficient Gr-1+CD11b+ cells sorted from peripheral blood of 4T1 tumor-bearing *Tgfb2*^{Flox} and *Tgfb2*^{MyeKO} mice. The samples were combined from 2-3 mice, with duplicates for each sample. Semi-quantitative data of normalized dot density is shown on the left. **(c)** CCL9 ELISA of myeloid cells sorted from peripheral blood of multiple 4T1 tumor-bearing *Tgfb2*^{Flox} and *Tgfb2*^{MyeKO} mice. **(d)** CCL9 expression of Gr-1+CD11b+ cells when cultured in 4T1 tumor-conditioned media with TGF-β neutralizing antibody 1D11 or control antibody 13C4. *P<0.05, **P<0.01, ***P<0.001.

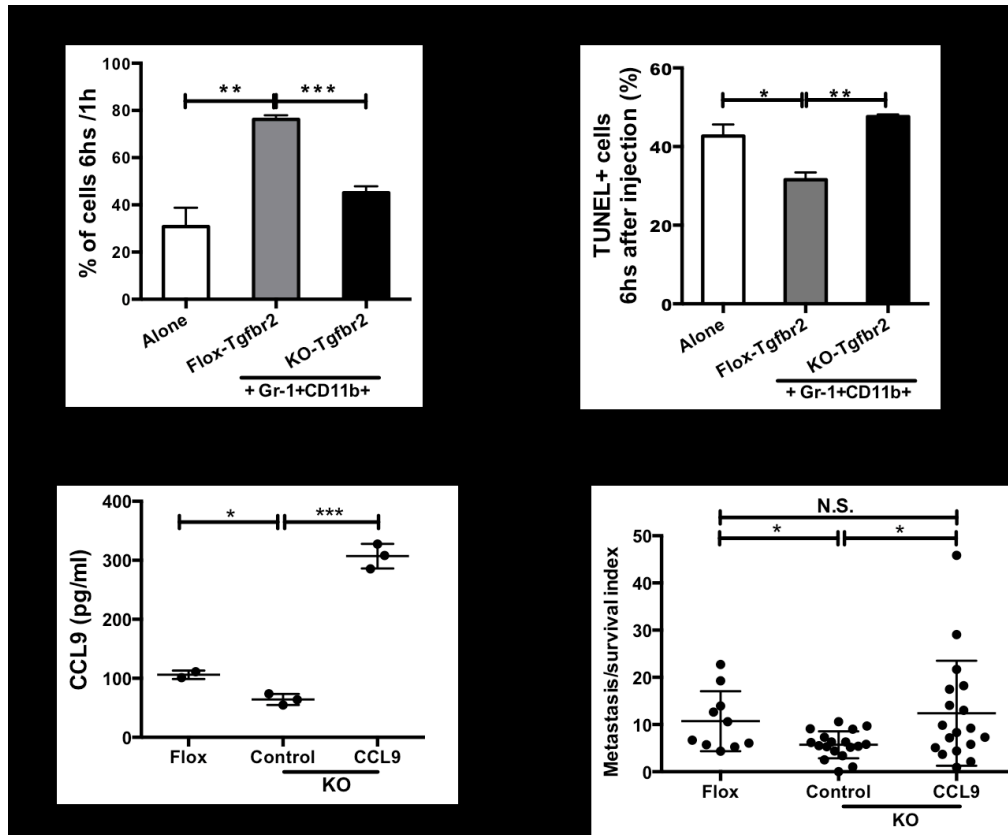


Figure 27. CCL9 overexpression in TβRII-deficient Gr-1+CD11b+ cells rescued the phenotype of decreased tumor cell survival and increased tumor cell apoptosis.

(a) SCVM for tumor cell survival. GFP labeled 67NR cells (5×10^5) were co-injected with floxed or TβRII-deficient Gr-1+CD11b+ cells (1×10^6). The lungs were taken out for imaging 1 and 6 hours after tail vein injection. Ten fields for each mouse lung were examined. Fluorescent signals at 6 hours were normalized to the 1 hour signal. $n=3$ mice per group. **(b)** TUNEL assay for tumor cell apoptosis from lung sections from (a). TUNEL positive cells were counted, calculated, and presented as percentages of GFP+ cells as shown at Y-axis. **(c)** ELISA showing overexpression of CCL9 in TβRII-deficient Gr-1+CD11b+ cells. **(d)** PUMA for tumor cell survival and metastasis. 67NR-GFP cells were co-injected with TβRII-deficient Gr-1+CD11b+ cells with or without CCL9 overexpression. Fluorescence images were obtained 14 days after lung section culture. Fluorescence signal per field was quantified then normalized to the day 0 signal and presented as metastasis survival index. Three mice per group, 3-4 lung sections per mouse. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

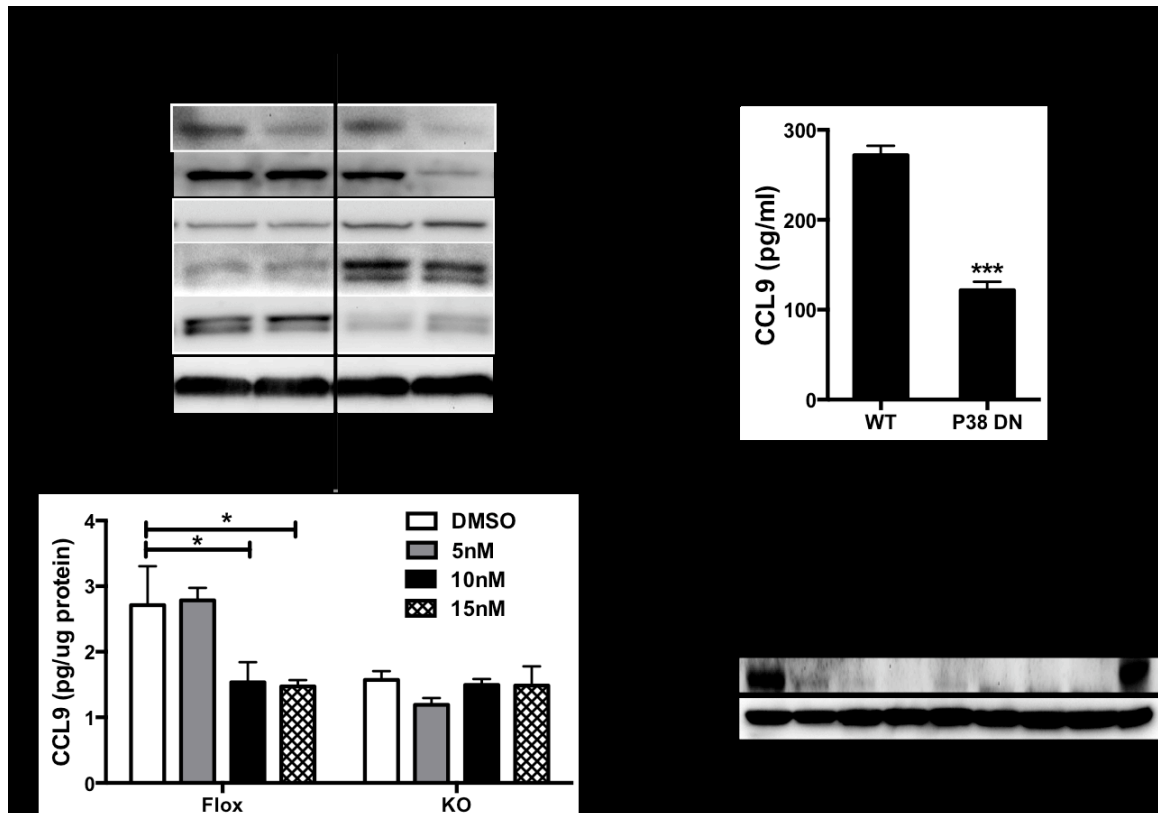


Figure 28. P38 mediated TGF- β signaling to promote CCL9 expression.

(a) Western bolts showing protein expressions of T β RII, P-p38, p38, P-SMAD2, and SMAD2 in T β RII-deficient Gr-1+CD11b+ cells compared to control cells. **(b)** Left panel: CCL9 ELISA of floxed or T β RII-deficient Gr-1+CD11b+ cells treated with the p38 inhibitor SB203580 for 6 hours. Right panel: Western blots showing one of P-p38 target protein P-ATF, as evidence for the efficiency of the p38 inhibitor SB203580. **(c)** CCL9 ELISA of floxed or T β RII-deficient Gr-1+CD11b+ cells treated with p38 inhibitor for 6 hours. *P<0.05, ***P<0.001.

Discussion

TGF- β /p38 pathway regulates CCL9 expression

Using $Tgfb2^{MyeKO}$ mice, we detected a significantly lower CCL9 expression in T β RII-deficient compared to floxed control Gr-1+CD11b+ cells (Fig. 26). Other TGF- β family members were also reported to regulate CCL9 expression. For example, deletion of bone morphology protein receptor II (BMPRII) in mammary epithelial cells increased CCL9 expression (Owens et al., 2012), and one allele deletion of SMAD4 in colon epithelial cells significantly increased CCL9 expression (Kitamura et al., 2007). Our study was consistent with previous publications that TGF- β family members regulated CCL9 expression.

We found that myeloid TGF- β promoted CCL9 expression, as deletion of T β RII in myeloid cells decreased CCL9 expression (Fig. 26). However, both previous publications stated that epithelial TGF- β signaling inhibited CCL9 expression, as deletion of BMPRII or one allele deletion of SMAD4 promoted CCL9 expression (Kitamura et al., 2007; Owens et al., 2012). The opposite TGF- β regulation of CCL9 expression in these two cell types was consistent with the fact that epithelial TGF- β inhibits metastasis, whereas myeloid TGF- β promotes metastasis. Activation of canonical SMAD-dependent or non-canonical SMAD-independent TGF- β downstream pathways might be one explanation for why myeloid TGF- β was tumor-promoting, whereas epithelial TGF- β was tumor-suppressing.

CCL9 expression was regulated by P-p38 in the non-canonical TGF- β pathway. Inhibition or knockdown of p38 blocked CCL9 expression (Fig. 28). This agreed with previous publications that p38 induced CCL9 by other factors, including fusion gene BCR-ABL, CpG oligodeoxynucleotides, and tumor necrosis factor-associated factor 6 (Iotti et al., 2007; Ravindran et al., 2010; Yang et al., 2008b).

CCL9 is an important effector in myeloid TGF- β function

Chemokines have been implicated in TGF- β regulation of tumor microenvironment and tumor progression. Loss of TGF- β signaling in carcinoma cells enhances chemokine production and correlates with poor prognosis in human breast cancer (Massague, 2012). In mouse models, deletion of TGF- β signaling in cancer epithelial cells increases metastasis due to chemokine expression, which promotes the interaction of carcinoma cells and bone marrow derived cells, cell migration, decreases tumor cell apoptosis and increases tumor cell survival (Bierie and Moses, 2006; Ijichi et al., 2011).

Our data demonstrated that CCL9 was the key regulator of the metastasis-promoting effects of myeloid TGF- β signaling, as overexpression of CCL9 in T β RII-deficient myeloid cells rescued tumor cell survival and metastatic capability (Fig. 27). Our data also provided a new understanding of tumor-host interactions in TGF- β regulation of metastasis.

Targeting CCL9 for metastasis treatment may bypass the unwanted or adverse effect of TGF- β neutralization.

Higher CCL9 expression either causes or correlates with higher metastasis. TGF- β signaling and biological functions are complex because of its dual roles, both as a tumor promoter and a suppresser. Thus, neutralization of TGF- β for cancer treatment may have adverse effects. A more feasible treatment might target the downstream effector CCL9, which may overcome the complexity of TGF- β pathway.

CHAPTER VI

SUMMARY AND CONCLUDING REMARKS

Tumor-host interaction is indispensable for metastasis. A number of studies indicate that hematopoietic cells, especially myeloid cells play a fundamental role in forming a premetastatic niche (Hiratsuka et al., 2006; Kaplan et al., 2005). In parallel with these studies, we have found a significant increase in Gr-1+CD11b+ cells that are recruited into the premetastatic lung. These immature myeloid cells produce and secrete cytokines/chemokines to build up an inflammatory, proliferative, and tumor-favorable premetastatic microenvironment prior to tumor cell arrival at the secondary organ (Yan et al., 2010). The tumor-promoting effect of Gr-1+CD11b+ cells acts under the regulation of TGF- β signaling. Our previous study has revealed that distinct from other cell types, myeloid specific TGF- β is a tumor promoter, since deletion of T β RII significantly decreased metastasis (Pang et al., 2013).

In this dissertation research, efforts have been made to understand how Gr-1+CD11b+ cells in the premetastatic organ facilitate tumor cell metastasis. In particular, we investigate (1) whether this assistance is through cytokine/chemokine expression, (2) whether it promotes tumor cell survival as one the most important rate-limiting steps in metastasis, and (3) whether it is under TGF- β regulation.

Using cytokine protein array screening, we found that CCL9 was significantly expressed and secreted by Gr-1+CD11b+ cells upon the co-culture with tumor cells, as well as in the premetastatic lung (Fig. 5, 6, and 8). CCL9 promoted tumor cell survival,

colonization and the resulting metastasis (Fig. 16, 20, 21, 23, and 24) through its only receptor CCR1 (Fig. 17 and 18). Mechanistically, CCL9 inhibited the PARP-dependent apoptosis pathway and promoted survival molecules including p-AKT and BCL-2 (Fig. 24). Importantly, CCL9 mediated TGF- β signaling in myeloid cells to promote metastasis, as deletion of T β RII on Gr-1+CD11b+ cells significantly decreased CCL9 expression and lung metastasis (Fig. 26). The underlying molecular mechanism involved p38, since inhibition or deletion of p38 significantly decreased CCL9 expression (Fig. 28). The autocrine effect of CCL9/CCR1 further promoted CCL9 expression and the survival of Gr-1+CD11b+ cells (Fig. 25). Importantly, CCL23, the human orthologue of mouse CCL9, and its receptor CCR1, correlated with the clinical stage and progression of breast and lung cancers, indicating human relevance (Fig. 12 and 13).

We report here the first time that CCL9 was significantly induced in immature myeloid cells in the premetastatic lung of tumor-bearing mice. It functioned as a tumor cell survival factor in the distant premetastatic site. CCL9 was also a critical downstream effector of the metastasis-promoting effect of myeloid-specific TGF- β signaling (Fig. 29).

Our work suggests a novel cellular and molecular mechanism underlying the “seed and soil” hypothesis, and identifies a unique target that contributes to the rate-limiting step of cancer cell survival. Our work also provides a new understanding of tumor-host interaction in TGF- β regulation of metastasis, and uncovers a downstream molecule of the metastasis-promoting effect of myeloid TGF- β signaling. Targeting CCL9 derived from myeloid cells might be a way to reprogram the premetastatic organ

microenvironment and consequently inhibiting metastasis. Additionally, targeting CCL9 as a downstream effector of the pro-metastatic myeloid TGF- β may overcome some of the negative consequences of TGF- β neutralization.

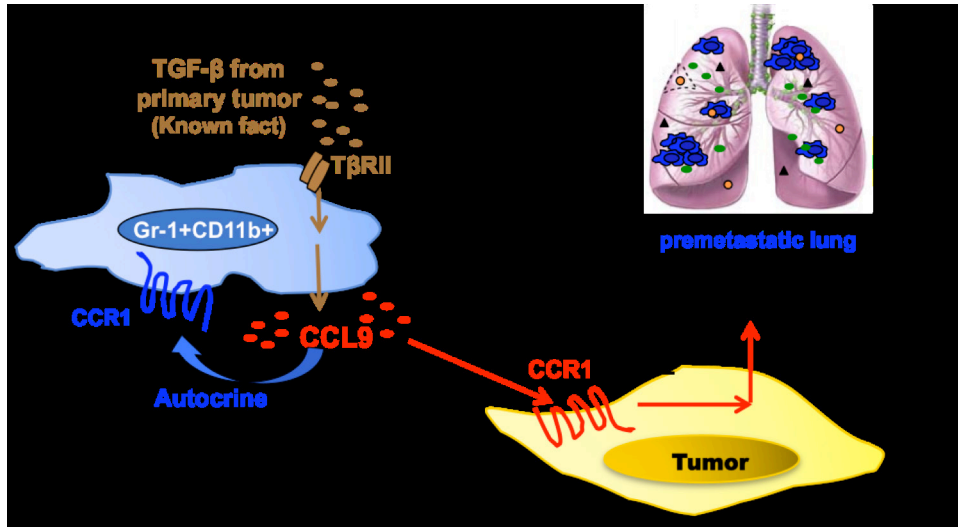


Figure 29. Schematic hypothesis.

TGF-β produced by the primary tumor and myeloid cells signals through TβRII on myeloid cells to stimulate CCL9 production in myeloid cells (step# 1). CCL9 then signals through CCR1 on tumor cells and enhances tumor cell survival and metastatic colony formation (step# 2). CCL9 also signals through the CCR1 receptor on myeloid cells and mediates an autocrine effect that increases CCL9 production and myeloid cell survival (step# 3).

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CURRICULUM VITA

Education

Doctor of Philosophy, Ph.D., Johns Hopkins University, 2009 - 2015

National Institutes of Health- Johns Hopkins University, Cell, Molecular, Developmental Biology & Biophysics Graduate Partnership Program (NIH-JHU CMDB GPP), Baltimore and Bethesda, Maryland

Bachelor of Science, B.S., Major in Biotechnology, 2003 - 2007

College of Life Science and Technology, Huazhong University of Science and Technology (HUST, a top ten university in China), Wuhan, Hubei, China (GPA: 80.23/100, 173.5 credits)

Employment History

Li Yang Lab, LCBG (Laboratory of Cancer Biology and Genetics), CCR (Center for Cancer Research), NCI (National Cancer Institute), NIH (National Institutes of Health), Bethesda, MD Ph. D. Candidate / NIH-JHU GPP CRTA fellow, 2009 - 2015

- First-author of a Cancer Research paper. Develop, manage, coordinate, conduct, and perform this research project.
- Fifth-author of a Cancer Discovery paper. Organize, coordinate, collaborate, and perform this research project.
- Currently lead two projects, one manuscript rebuttal and one manuscript in preparation.
- Train, track, and oversee postdoctoral fellows, graduate students, and summer students.
- Review and evaluate papers/protocols/grants. Recommend and determine suggestions for improvement or acceptance.
- Develop, evaluate, prepare, and conduct research projects/protocol independently.
- Gather, analyze, track, and manage research data to develop and determine future research directions.
- Personally coordinate and communicate with collaborators from other labs to test scientific hypotheses.
- Review, present, and report data at international conferences, including AACR Cellular Heterogeneity in the Tumor Microenvironment 2014, AACR Annual meeting 2013, and Cold Spring Harbor meeting 2012.

Biochemistry/Developmental Biology Lab Class, Johns Hopkins University, Baltimore, MD

Teaching Assistance (TA), Spring and Fall Semester 2009 - 2010

- Develop, organize, and prepare experiments for undergraduate students.
- Explain, review, and demonstrate each lab experiment to students.
- Review and communicate with students about principles. Provide solution to any issues that may occur.
- Coordinate and communicate with professors and TAs to review and evaluate lab reports/exams, as well as oversee and proctor exams.

Li Yang Lab, LCBG, CCR, NCI, NIH, Bethesda, MD

Cancer Research Training Award Trainee, 1/2009 - 9/2009

- Develop, manage, coordinate, and conduct a research project that evaluates the host immunity in lung metastasis.

- Lead, coordinate, and conduct the establishment of a new lab.
- Develop, prepare, present, prepare, and establish standard lab protocols.
- Train, track, and oversee new postdoctoral fellows.
- Evaluate, determine, recommend, order, prepare, track, and organize research equipment, reagents, and supplies.

**Harold Moses Lab, Vanderbilt University, Nashville, Vanderbilt-Ingram Cancer Center, TN
Research Assistant, 2008 - 2009**

- Conduct, perform, and assist in a research project that evaluates the host immunity in lung metastasis.
- Communicate and coordinate with research fellows and graduate students.
- Report and present data in lab meetings.
- Identify, recommend, and prepare samples, reagents, and documents for a new lab.

**Qing Wang Lab, Department of human genetics, HUST, China
Undergraduate Thesis, 01/2007-06/2007**

- Manage, track, conduct, perform, evaluate and analyze an independent research project that successfully identifies a new human epilepsy mutation in one family by gene sequencing.
- Coordinate, communicate, report, and present research progress and experimental data to thesis mentor.

**Robert L. McDonald Lab, Department of Neurology, Vanderbilt University, Nashville, TN
Summer Internship, 07/2005-08/2005 and 07/2006-08/2006**

- Conduct, perform, evaluate, and analyze the effect of a new mutation in epilepsy.
- Communicate, review, report, and present experimental data to lab manager.

Awards and Honors

- NIH Summer Research Mentor Award, NIH, 2014
- Fellows Award for Research Excellence (FARE) and \$1000 travel award, NIH, 2014
- Cancer Research Training Award (CRTA), NIH, 2009 to present
- Frist level Award for Undergraduate Dissertation Research, HUST, 2007
- Honor of Merit from Dow Jones Newswires, 2006
- Third level Award for HUST Undergraduate Students Thesis, HUST, 2005
- Award and scholarship for research internship, HUST, 2005
- Award and scholarship of Excellent Student Leader, HUST, 2003-2004
- First level prize of College English stage play, HUST, 2003

Publications

Yan HH, Pang Y, Achyut BR, Lizard M, Hunter K, Khanna C, Hollander C, Yang L. CCL9 induced by TGF- β signaling in myeloid cells enhances tumor cell survival in the premetastatic organ. *Cancer Discovery*. Rebuttal (IF: 15.929)

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Professional Associate

- American Association of Cancer Research (AACR), 2009 – present
- Social of Chinese Bioscientists in America (SCBA), 2012- present

Other Activities

- **Lab management 2009-present.** Track, identify, and order supplies. Manage equipment. Organize meetings.
- **Judge for FARE 2015 Travel Award Competition 2014.** Review, evaluate, and determine top research abstracts.
- **Organizer for LCBG Annual celebration 2012-2014.** Organize, prepare, and conduct activities.
- **Member of graduate student council (GSC) 2009-2010.** Organize seminars as a Thesis Committee member.
- **Member of VUCSSA (Vanderbilt University Chinese Students and Scholars Association) 2007-2008.** Lead, organize, coordinate, and prepare 2008 Beijing Olympic Festival and 2008 Chinese New Year Festival.
- **Leader of college student union 2003-2006.** Coordinate, organize, and conduct college activities.
- **Leader of class 2003-2007.** Lead, coordinate, and organize class meetings and activities. Plan and organize a trip of 14 classmates to a village in western China to teach primary school students.